Autophagy as a cell fate determinant facing drugs and trophic-nutritional deprivation

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Lleida, 2016

To my Family

Ackwoledgement

It has been a long journey, an Odyssey from a certain point of view and I am very glad I am finally able to write my acknowledgements.

My first thanks go to my supervisors, Prof. Judit Ribas and Prof. Jacint Boix. It was an honour to work in your lab. Thanks for teaching me how to make science, for the opportunity to express my creativity, for the guidance, the discussions and exchange of views. This all helped me very much to find the solutions for the difficulties and paradoxes I encountered during my research.

I would also like to acknowledge AGAUR for the financial support to pursue my doctoral thesis.

I am very much grateful to all the previous members of the lab. Carmen C., Thanks for welcoming in a new city and make me feel at home. Ares, thanks for the Italian chats and funny moments. Nuria and MariCarmen, thanks for the support. Juan, thanks for the talks about football and Topgear.

Thanks to all the Professor and members of the IRB Lleida that I met during these years. Thanks particularly to Prof. C. Cantì, Prof. Mª. de la Torre, Nati, Deepshikha, Charu, Upasana, Disha, Ana Macia, Nuria E., Venky, Vicky, Marta C., Mireia, Ambika, Marta V., David A., Saravanan, Daniel, Alba, Maria Josè.

A big thanks to Montse, Marta and Imma for the encouragement and laughs. A special word of appreciation to Lidia and Anais for their help and suggestions.

Thanks to my friends and acquaintances for the support, the help and the criticism about the cover. A particular thanks to Dominika who stood by me in a very difficult moment. My heartfelt thanks to Laura B. and Ulviyya for their support.

An immense gratitude to my family for the neverending support, understanding and encouragement. This PhD thesis is dedicated to them.

Index

I ABBREVIATIONS

3-MA	3-methyladenine
A-CDase	Acid ceramidase
AIF	Apoptosis Inducing Factor
ALDh4	Aldehyde Dehydrogenase 4
A-SMase	Sphingomyelinase
AMPK	Monophosphate-activated Protein Kinase
ANT	Adenine Nucleotide Translocase
ATF6	Activating Transcription Factor 6
ATM	Ataxia Telangiectasia Mutated
ATGs	Autophagy Proteins
BIR	Baculovirus IAP Repeat
BCL-2	B-Cell CLL/Lymphoma 2
BID	BH3-Interacting Domain death agonist
BSO	L-Buthionine-Sulfoximine
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
c-FLIP	Cellular FLICE-like inhibitory proteints
CHK1	Check point kinase 1
CL	Cardiolipin
CMA	Chaperone-mediated autophagy
CRT	Calcireticulin
CPT	Camptothecin
CsA	Cyclosporin A

CYLD	Cylindromatosis
CYPD	Cyclophilin D
Cytochrome C	Cyt C
DAP1	Death associated protein
DAMPs	Damage-associated molecular patterns
DCC	Deleted in colorectal cancer
DCFDA	Dichlorofluorescin diacetate
DED	Death effector domain
DIF-1	Differentiation inducing factor-1
DIABLO	Direct IAP binding protein with low pI
DDR	DNA damage response
DR	Death receptors
DRAM	Damage-regulated autophagy modulator
Drp1	Dynamin-related protein 1
DUBs	Deubiquitinating enzymes
EndoG	Endonuclease G
ERAD	ER-associated protein degradation
Eto	Etoposide
FADD	Fas-associated DD
FADH	Flavin adenine dinucleotide
GAP	GTPase activating protein
GF	Growth factors
GPX	Glutathione peroxidase
HECT	Homologous to the E6-AP Carboxyl Terminus
HMGB1	High mobility group protein B1
HOSE	Human ovarian surface epithelial
HSF-1	Heat shock transcription factor 1

HSP90	Heat shock protein 90
HSP70	Heat shock protein 70
ICAD	Inhibitor of caspase-activated deoxyribonuclease
IAPs	Inhibitors of apoptosis
IF	Intermediate filaments
IRE1	Inositol-requiring protein 1
IRI	Ischemia reperfusion injury
JNK	c-Jun N-terminal kinase
LMP	Lysosomal membrane permeabilization
LOX	Lipoxygenase
IMM	Inner mitochondrial membrane
MDM2	Mouse double minute 2 homolog gene
MLKL	Mixed lineage kinase domain-like
MnSOD	Manganese superoxide dismutase
MOMP	Mitochondrial Outer Membrane Permeabilization
MPTP	Mitochondrial permeability transition pore
NAF-1	Nutrient-deprivation autophagy factor-1
NCX	Na+/Ca2+ exchanger
NEF	Nucleotide exchange factor
NAD	Nicotinamide adenine dinucleotide
NLRs	NOD-like receptors
NOXs	NADPH oxidases
NQO1	Quinone oxidoreductase
OMM	Outer mitochondrial membrane
OXPHOS	Oxidative phosphorylation
PAMPs	Pathogen-associated molecular pattern
PARP-1	Poly(ADP-ribose) polymerase 1

PERK	Protein kinase RNA-like ER kinase
PHD	Plant homeodomain
PUFAs	Polyunsaturated fatty acids
PES	Phenylethinesulfonamide
PI3K	Phosphoinositol-3-kinase
PIPs	Phosphatidylinositol phosphate lipids
PMCA	Plasmatic Ca2+ pump
PPP	Pentose phosphate pathway
PtdIns3P	Phosphatidylinositol 3-phosphate
RHEB	Ras homolog enriched in brain
RIPK1	Receptor-interacting protein kinase 1
RING	Really interesting new gene
SCO2	Cytochrome oxidase 2
SERCA	Sarco(endo)plasmic reticulum Ca2+ ATPase
SHRs	Steroid hormone receptors
SM	Starvation media
SMAC	Mitochondria-derived activator of caspases
SNAREs	Soluble NSF attachment protein receptors
SOD	Superoxide dismutase
SSB	Single-strand DNA breaks
STP	Staurosporine
TAB2	TAK1 binding protein 2
TAD	Transactivation domain
TCA	Tricarboxylic acid
TIGAR	TP53-induced glycolysis and apoptosis regulator
TLRs	Toll-like receptors
TNF	Tumor necrosis factor

TRADD	TNF-R1-associated death-domain protein
TRAIL	TNF-related apoptosis inducing ligand
TSPO	Translocator protein
ULK	Unc-51-line kinase
UPR	Unfolded protein response
Ub	Ubiquitin
UPS	Ubiquitin proteasome system
UVB	Ultraviolet B
VDACs	Voltage Dependent Anion Channels

II ABSTRACT

Title: Autophagy as a cell fate determinant facing drugs and trophic-nutritional deprivation

1 Abstract

Cell death is an indispensable event during mammalian lifespan. A morphological classification defined apoptosis, necrosis and autophagy as the three main types of cell death. Apoptosis (type I) is characterized by a mechanism of cell suicide adopted by multicellular organisms to shape organs and tissues. In addition, apoptosis is commonly engaged in response to traditional radio- and chemotherapy treatments of cancer. Autophagy is the main lysosomal degradative program for the turnover of organelles, long-living proteins and the generation of metabolites during cell conditions of starvation. Autophagy represents also a means of cell suicide (type II or autophagic cell death). Moreover, autophagy can be placed upstream of other modalities of cell death. Finally, necrosis (type III) is characterized by the disruption of the plasma membrane in response to accidental cues or, alternatively, to the activation of genetically-regulated pathways.

In the first part of our study, we analyzed the contribution of autophagy to the cell death in several cell lines exposed to a complete trophic and nutritional deprivation by means of a specific starvation medium (SM). The cell cultures deprived of growth factors, glucose and other nutrients mimic the most severe scenario in an insufficiently vascularized tumor. Deprived cells responded by triggering autophagy and apoptosis by the intrinsic pathway. In this experimental paradigm, autophagy accomplished a dual role. At short times, it primes the mitochondrial outer membrane permeabilization (MOMP). In cells subjected to SM, the inhibition of canonical autophagy by means of genetics (*ATG 5* -/- and Beclin-1 siRNA) or pharmacology with 3-methyladenine (3-MA) limited MOMP, the release of cytochrome C and the subsequent activation of caspases. However, at long times, the pro-survival function of autophagy prevailed, thus underscoring the temporal frame of the phenomena. In addition, starved cells subjected to pro-apoptotic drugs such as staurosporine, camptothecin or etoposide were protected from apoptosis if autophagy was either genetically or pharmacologically disabled. These observations are relevant for the better understanding of the response to anticancer drugs of solid tumors with a deficient irrigation.

Phenylethinesulfonamide (PES), also known as Pifithrin-μ, is known for its ability to disrupt some homeostatic networks such as the proper refolding of proteins by HSP70, autophagy, the ubiquitin proteasome system (UPS) and the translocation of p53 to mitochondria. PES has raised a great interest because of its lethal selectivity for cancer cells. In our cell models, PES induced a non-necroptotic regulated-type of necrosis that depended on the partnership of p53 and reactive oxygen species (ROS). Accordingly, p53-deficiency or thiol antioxidants protected cells in culture from PES-induced cell death. Consistently, the depletion of glutathione by means of L-buthionine-sulfoximine (BSO) sensitized cells to PES. Therefore, BSO association with PES defined a promising synergism envisaging cancer therapy. Unexpectedly, *Bax* $^{-/-}$ and *Bax* $^{-/-}$/*Bak* $^{-/-}$ (DKO) MEFs displayed greater sensitivity to PES-elicited necrosis than their wild-type (WT) counterparts. Reintroduction of a functional BAX protein protected BAX deficient cells from PES cytotoxicity. This fact was indicating a pro-survival function in BAX that is rarely observed. The presence of increased mitochondrial fusion in WT MEFs, those with increased resilience, suggested a potential role for mitochondrial dynamics in the mode of action of PES. This was proved by means of Mdivi-1, a chemical inducer of mitochondrial fusion. Cells with fused mitochondria were better tolerating the treatments with PES. Moreover, BAX deletion minimized the protective effect of Mdivi-1, thus suggesting the involvement of BAX in promoting mitochondrial fusion.

In conclusion, in the context of trophic and nutritional deprivation (SM), we have identified time as the determinant factor in the autophagy-mediated transition from a pro-death apoptotic program to a pro-survival one. In addition, we have investigated the lethal mode of action of PES and uncovered a feed-back network comprising p53, ROS, BAX and mitochondrial dynamics.

Títol: L'autofàgia com a determinant del destí cel·lular front a fàrmacs i privació tròfica-nutricional

2 Resum

La mort cel·lular és un esdeveniment indispensable al llarg de la vida dels mamífers. Una classificació morfològica definia apoptosi, necrosi i autofàgia com els tres principals tipus de mort cel·lular. L'apoptosi (tipus I) es caracteritza per un mecanisme de suïcidi cel·lular que ha estat adoptat pels organismes multicel·lulars per a estructurar el òrgans i els teixits. A més, l'apoptosi és generalment activada en resposta als tractaments convencionals de ràdio- i quimioteràpia del càncer. L'autofàgia és el principal programa de degradació lisosomal per al reciclatge dels orgànuls, les proteïnes de llarga vida i la generació de metabòlits en condicions de privació nutricional de les cèl·lules. La autofàgia representa també un mecanisme suïcida cel·lular (el tipus II o de mort cel·lular per autofàgia). És més, l'autofàgia es pot situar apicalment regulant altres modalitats de mort cel·lular. Finalment, la necrosi (el tipus III) es caracteritza per la disrupció de la membrana plasmàtica en resposta a situacions accidentals o, alternativament, a l'activació de vies regulades genèticament.

A la primera part del nostre estudi, hem analitzat la contribució de l'autofàgia a la mort cel·lular en diverses línies de cèl·lules exposades a una privació completa tròfica i nutricional mitjançant un medi de cultiu específic (SM). Els cultius cel·lulars privats de factors de creixement, la glucosa i altres nutrients imiten l'escenari de major severitat dintre d'un tumor insuficientment vascularitzat. Les cèl·lules privades responien mitjançant l'activació de l'autofàgia i l'apoptosi per la via intrínseca. En aquest paradigma experimental, l'autofàgia complia una doble funció. A temps curts, promovia la permeabilització de la membrana externa mitocondrial (MOMP). En cèl·lules sotmeses a SM, la inhibició de l'autofàgia canònica per mitjans genètics (*Atg 5* $^{-/-}$ i Beclina-1 siRNA) o farmacològics amb 3-metiladenina (3-MA) limitava la MOMP, l'alliberament de citocrom C i la subseqüent activació de les caspases. En canvi, a temps llargs, la funció pro-supervivència de l'autofàgia prevalia, emfatitzant-se així el marc temporal dels fenòmens. Addicionalment, les cèl·lules sota privació sotmeses a fàrmacs pro-apoptòtics com ara estaurosporina, camptotecina o etopòsid eren protegides de l'apoptosi si l'autofàgia estava interferida genèticament o farmacològica. Aquestes observacions són rellevants per entendre millor la resposta als medicaments anticancerosos dels tumors sòlids amb irrigació insuficient.

La feniletísulfonamida (PES), també coneguda com Pifithrina-μ, és coneguda per la seva capacitat d'alterar algunes xarxes homeostàtiques com ara el correcte replegament de proteïnes per HSP70, l'autofàgia, el sistema ubiqüitina-proteosoma (UPS) i la translocació de p53 a les mitocòndries. PES desperta un gran interès per la seva letalitat selectiva cap a les cèl·lules canceroses. En els nostres models cel·lulars, PES va induir un tipus de necrosi, regulat i no necroptotic que depenia de l'associació de p53 i espècies reactives d'oxigen (ROS). En conseqüència, la deficiència en p53 o els antioxidants tiòlics protegien les cèl·lules en cultiu de la mort cel·lular induïda per PES. En coherència, l'esgotament de glutatió per mitjà de L-butionina-sulfoximina (BSO) sensibilitzava les cèl·lules front a PES. En conseqüència, l'associació de BSO amb PES definia un sinergisme prometedor de cara a la teràpia del càncer. Inesperadament, *Bax* $^{-/-}$ i *Bax* $^{-/-}$ més *Bak* $^{-/-}$ (DKO) MEFs mostraven una major sensibilitat a la necrosi provocada per PES que la seva variant salvatge (WT). La reintroducció d'una proteïna BAX funcional a les cèl·lules deficients en BAX protegia de la citotoxicitat de PES. Aquest fet indicava una funció de pro-supervivència en BAX que rarament s'observa. La presència d'un increment en la fusió de les mitocòndries als WT MEFs, que mostren una major resistència, va suggerir un potencial paper de la dinàmica mitocondrial en el mecanisme d'acció de PES. Això es va demostrar per mitjà de Mdivi-1, un inductor químic de fusió mitocondrial. Les cèl·lules amb mitocòndries fusionades toleraven millor els tractaments amb PES. És més, el dèficit de BAX minimitzava l'efecte protector de Mdivi-1, fet que suggeria la participació de BAX en promoure la fusió mitocondrial.

En conclusió, en el context de la privació tròfic i nutricional (SM), hem identificat el temps com el factor determinant pel qual l'autofàgia genera la transició d'un programa pro-apoptòtic de mort a un de pro-supervivència. Addicionalment, hem investigat el mecanisme d'acció letal de PES i descobert una xarxa de retroalimentacions que inclou a p53, ROS, BAX i la dinàmica mitocondrial.

Título: La autofagia como determinante del destino celular frente a fármacos y privación trófico-nutricional

3 Resumen

La muerte celular es un evento indispensable a lo largo de la vida de los mamíferos. Una clasificación morfológica definía apoptosis, necrosis y autofagia como los tres principales tipos de muerte celular. La apoptosis (tipo I) se caracteriza por un mecanismo de suicidio celular que ha sido adoptado por los organismos multicelulares para estructurar los órganos y los tejidos. Además, la apoptosis es generalmente activada en respuesta a los tratamientos convencionales de radio- y quimioterapia del cáncer. La autofagia es el principal programa de degradación lisosomal para el reciclaje de los orgánulos, las proteínas de larga vida y la generación de metabolitos en condiciones de privación nutricional de las células. La autofagia representa también un mecanismo suicida celular (el tipo II o de muerte celular por autofagia). Es más, la autofagia puede situarse apicalmente regulando otras modalidades de muerte celular. Finalmente, la necrosis (el tipo III) se caracteriza por la disrupción de la membrana plasmática en respuesta a situaciones accidentales o, alternativamente, a la activación de vías reguladas genéticamente.

En la primera parte de nuestro estudio, hemos analizado la contribución de la autofagia en la muerte celular de varias líneas de células expuestas a una privación completa trófica y nutricional mediante un medio de cultivo específico (SM). Los cultivos celulares privados de factores de crecimiento, la glucosa y otros nutrientes imitan el escenario de mayor severidad dentro de un tumor insuficientemente vascularizado. Las células privadas respondían mediante la activación de la autofagia y la apoptosis por la vía intrínseca. En este paradigma experimental, la autofagia cumplía una doble función. A tiempo cortos, promovía la permeabilización de la membrana externa mitocondrial (MOMP). En células sometidas a SM, la inhibición de la autofagia canónica por medios genéticos (*Atg 5* $^{-/-}$ y Beclina-1 siRNA) o farmacológicos con 3-metiladenina (3-MA) limitaba la MOMP, la liberación de citocromo C y la subsecuente activación de las caspasas. En cambio, en tiempos largos, la función pro-supervivencia de la autofagia prevalecía, enfatizando así el marco temporal de los fenómenos. Adicionalmente, las células bajo privación sometidas a fármacos pro-apoptóticos como estaurosporina, camptotecina o etopósido eran protegidas de la apoptosis si la autofagia estaba interferida genética o farmacológicamente. Estas observaciones son relevantes para entender mejor la respuesta a los medicamentos anticancerosos de los tumores sólidos con irrigación insuficiente.

La feniletinosulfonamida (PES), también conocida como Pifithrina-μ, es conocida por su capacidad de alterar algunas redes homeostáticas como el correcto repliegue de proteínas por HSP70, la autofagia, el sistema ubiquitina-proteosoma (UPS) y la translocación de p53 a las mitocondrias. PES despierta un gran interés por su letalidad selectiva hacia las células cancerosas. En nuestros modelos celulares, PES indujo un tipo de necrosis, regulado y no necroptótico que dependía de la asociación de p53 y las especies reactivas de oxígeno (ROS). En consecuencia, la deficiencia de p53 o los antioxidantes tiólicos protegían las células en cultivo de la muerte celular inducida por PES. En coherencia, el agotamiento de glutatión por medio de L-butionina-sulfoximina (BSO) sensibilizaba las células frente a PES. En consecuencia, la asociación de BSO con PES definía un sinergismo prometedor de cara a la terapia del cáncer. Inesperadamente, Bax $^{-/-}$ y Bax $^{-/-}$ más Bak $^{-/-}$ (DKO) MEFs mostraban una mayor sensibilidad a la necrosis provocada por PES que su variante salvaje (WT). La reintroducción de una proteína BAX funcional a las células deficientes en BAX protegía de la citotoxicidad de PES. Este hecho indicaba una función pro-supervivencia en BAX que raramente se observa. La presencia de un incremento en la fusión de las mitocondrias en los WT MEFs, que muestran una mayor resistencia, sugirió un potencial papel de la dinámica mitocondrial en el mecanismo de acción de PES. Esto se demostró mediante Mdivi-1, un inductor químico de fusión mitocondrial. Las células con mitocondrias fusionadas toleraban mejor los tratamientos con PES. Es más, el déficit de BAX minimizaba el efecto protector de Mdivi-1, lo que sugería la participación de BAX en promover la fusión mitocondrial.

En conclusión, en el contexto de la privación trófico y nutricional (SM), hemos identificado el tiempo como el factor determinante por el que la autofagia genera la transición de un programa pro-apoptótico de muerte a uno de pro-supervivencia. Adicionalmente, hemos investigado el mecanismo de acción letal de PES y descubierto una red de retroalimentaciones que incluye a p53, ROS, BAX y la dinámica mitocondrial

III PREFACE

Cell Death on the verge of Biology and Philosophy

“Se la morte, signor mio, fosse come uno di quegli insetti strani, schifosi, che qualcuno inopinatamente ci scopre addosso... Lei passa per via; un altro passante, all'improvviso, lo ferma e, cauto, con due dita protese le dice: «Scusi, permette? lei, egregio signore, c’ ha la morte addosso ». E con quelle due dita protese, la piglia e la butta via... Sarebbe magnifica! Ma la morte non è come uno di questi insetti schifosi. Tanti che passeggiano disinvolti e alieni, forse ce l'hanno addosso; nessuno la vede; ed essi pensano quieti e tranquilli a ciò che faranno domani e doman l'altro.”

L’uomo dal fiore in bocca. **Luigi Pirandello**.

"Death is the turn of the road, dying is only not be seen." **Fernando Pessoa.**

Death stands there in the background, but don’t be afraid. Hold the watch down with one hand, take the stem in two fingers, and rotate it smoothly. Now another installment of time opens, trees spread their leaves, boats run races, like a fan time continues filling with itself, and from that burgeon the air, the breezes of earth, the shadow of a woman, the sweet smell of bread.

What did you expect, what more do you want? Quickly strap it to your wrist, let it tick away in freedom, imitate it greedily. Fear will rust all the rubies, everything that could happen to it and was forgotten is about to corrode the watch’s veins, cankering the cold blood and its tiny rubies. And death is there in the background, we must run to arrive beforehand and understand it’s already unimportant.

Cronopios and Famas. **Julio Cortázar.**

“With the exception of mankind, all creatures are immortal, for they know nothing of death.”

The Immortal, Aleph. **Jorge Luis Borges.**

“Have you ever wondered if death is the same for all living beings, be they animals, human beings included, or plants, from the grass you walk on to the hundred-meter-tall sequoiadendron giganteum, will the death that kills a man who knows he's going to die be the same as that of a horse who never will.”

Death at Intervals. **José Saramago,**

Under optimal growing conditions, most prokaryotes and unicellular eukaryotes are potentially immortal. Their immortality is justified by their mechanisms of asexual reproduction, which allows the generation of two identical offspring from a single parent cell, in a process termed binary fission. However, some studies have underscored the intriguing possibility that regulated cell death of unicellular organisms (e.g. bacteria, yeast) responds to an altruistic attempt to benefit the entire colony [1].

In multicellular organisms, the concept of altruistic cell death is utterly relevant. Cell death in this context is an indispensable event. In order to first, develop and later, maintain a healthy state, individual cells divide, specialize or commit suicide. In this scenario, death of cells offers undoubted beneficial effects for the survival of the organism they belong to.
The passage from uni- to multi-cellularity has driven an evolutionary pressure for the appearance of both genetically regulated cell death programs. The discovery of regulated cell death (RCD) dates to the pioneering studies of the nematode *Caenorhabditis elegans*. The investigation of genetic mutants from this worm revealed that cell death by apoptosis was contingent on the presence of a death machinery, which comprise 4 genes: an activator (*ced-4)*, an executioner (*ced-3*), a repressor of cell death (*ced-9*) and the antagonist of ced-9 (*egl-1)*. In higher eukaryotes, the cell death network has reached its acme of complexity and the three main mechanisms of cell death (apoptosis, necrosis and autophagic cell death) are controlled by more than 100 key genes.

Higher multicellular organisms are not immortal and their reproduction depends essentially on sexual mechanisms. Within the same species, the idea of the death of a single individual as an altruistic adaptation (for the greater benefit of the species as a whole), can be traced back to August Weismann and Charles Darwin, two of the most notable evolutionary theorists of the 19th century. Nevertheless when dealing with human beings, such a concept requires a more complex multidisciplinary pondering. Human being is in fact the only creature who strives within a paradox: an ethereal "symbolic identity" caged in an evanescent body. As the American anthropologist Ernest Becker wrote:

> "*Man is literally split in two: He has an awareness of his own splendid uniqueness in that he sticks out of nature with a towering majesty, and yet he goes back into the ground a few feet in order blindly and dumbly to rot and disappear forever." … "He is out of nature and hopelessly in it"* [1].

Such terrible conflict represents our existential background. All the other animals are spared this contradiction, for they live an unconsciousness and therefore timeless life. Existentialism is an attitude that dates back to ancient Greek philosophers, Heraclitus, Plato and Socrates and medieval thinkers such as Saint Augustin. Schopenhauer considered death as "the muse of philosophy" and the awareness of death - he continued - has been one of the strongest impulses to its birth [2].

Ancient Greek scholars, especially Plato and Socrates, primarily focused on the dichotomy "body versus mind" - that is, the relationship between physical reality and phenomenology - a theme that interested many following philosophers (Descartes, Kant and Husserl among others) and still challenges modern neurophysiology and artificial intelligence.

For many ancient and modern religions the death-rebirth cycle or the promise of an eternal life after death was and still represents the only possible existential answer.

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[1] Becker, Ernest (1973). *The Denial of Death*. New York: Simon & Schuster.

[2] Schopenhauer, Arthur (1909). *The World as Will and Idea.*

Philosophy has instead employed many logical stratagems to deny death or alternatively to soften its burden, but "*death turns its deaf ear to the invites of philosophy*" [3]. Some philosophical currents, like Stoicism and Epicureanism involved the devaluation of death but in an antithetical manner: the former hanging on life lack of meaning, the latter exalting it. Other philosophers, including for example Hegel, Spinoza, Feuerbach and Marx, had instead looked at the death of an individual within a sort of evolutionistic perspective.

Heidegger and Kierkegaard are considered the fathers of modern Existentialism. Their ideas, which focused on the concept of anguish, have influenced psychology, arts and literature. The term Existentialism was precisely coined by the French philosopher Jean-Paul Sartre and it was retroactively applied to philosophers of the 19th and 20th century, whose thinking was characterized by putting the existence ("Being") on top of the essence ("Nature"), reflecting on how "*The existence precedes the essence*". [4] Existentialism can be defined as the attempt to understand the interlinks between existence and consciousness.

The problem of existence and the ultimate and deep meaning of death has had such a strong impact in philosophy that Camus, in his famous absurdist essay "The Myth of Sisyphus" declares:

> "*There is but one truly serious philosophical problem, and that is suicide. Judging whether life is or is not worth living amounts to answering the fundamental question of philosophy. All the rest—whether or not the world has three dimensions, whether the mind has nine or twelve categories—comes afterwards. These are games; one must first answer*". [5]

Paraphrasing Camus' statement and passing from the general ("Being") to its particular ("Cell"), the same judgment can be stated regarding cellular death. In the context of a multicellular organism, the presence of an intrinsic death machinery to "judge" whether a cell is worth living or not, means the actual survival of the individual. Overall, the comprehension of the mechanisms by which a cell encounters its death is a fundamental challenge for medicine and a key for cancer, neurodegeneration, cerebral stroke, heart failure and autoimmune diseases therapy.

[3] Morin, Edgar (1976). *L'Homme et la Mort*.

[4] Sartre, Jean-Paul (1943). *Being and Nothingness.* Gallimard.

[5] Camus, Albert (1942). *The Myth of Sisyphus*. Gallimard.

IV INTRODUCTION

1 Classification of Cell Death

According to the recommendations of the Nomenclature Committee on Cell Death (NCCD) 2015, two are the main criteria to identify a dead cell: irreversible plasma membrane permeabilization and/or complete fragmentation of the cell, including its nucleus [2].

Cell death can be classified on the basis of its morphological, functional, immunological or biochemical features [3]. In the following sections, we will develop the aforementioned classifications.

1.1 Morphological classification of cell death

One of the first attempts to classify cell death dates back to the 70's when Schweichel and Merker described the morphological traits of cell demise during the fetal development of rats and mice [4]. The lysosomal involvement was determinant to propose the following three cell death modalities: type I or heterophagy (Apoptosis), type II or Autophagic cell death and type III or non-lysosomal cell death (Necrosis). In the 90's, this classification was revisited by Clarke, albeit maintaining the previous three developmental cell death paradigms [5].

Each cell death modality is characterized by a specific ultrastructural appearance (**Fig. 1**):

- Apoptosis is characterized by cytoplasmic shrinkage, chromatin condensation that classically initiates at the nuclear membrane (marginalization) and that is then extended to the whole nucleus (pyknonis), nuclear fragmentation (karyorrhexis), minimal perturbation of the organelles and the presence of a peculiar boiling-like ("blebbing") process, which ends up with the cellular fragmentation into sealed membrane vesicles, termed apoptotic bodies.
- Autophagic cell death is characterized by large-scale autophagic vacuolization of the cytoplasm.
- The main morphological change of necrotic cell death is the cellular swelling (oncosis), rounding of the cell, organelle swelling, translucent and structurally disorganized cytoplasm, a largely intact distended nucleus and, eventually, the plasma membrane rupture.

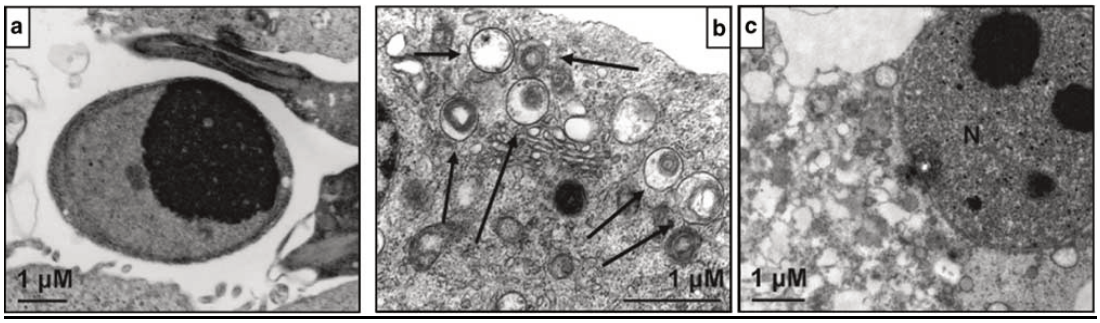

Figure 1: Morphological features of cell death by transmission electron microscopy. **a)** Type I or Apoptotic cell death morphology with nuclear shrinkage and pyknosis. **b)** Type II or autophagic cell death with extensive presence of double membrane cytoplasmic autophagolysosome (arrows), filled with cytoplasmic content and organelles. **c)** Type III or Necrosis with intact nucleus (N), massive dismantled cytoplasm and plasma membrane rupture. Adapted from Cell Death and Differentiation (2007) 14**,** 1237–1243.

1.2 Functional Classification of Cell death

On a functional basis, cell death can proceed through accidental or regulated manner. Accidental Cell death is triggered in response to a severe physical (e.g. high temperature), chemical (e.g. detergents or extreme change of pH) or mechanical (shearing) insult. It is characterized by its immediacy and insensitivity to any pharmacologic or genetic approach. On the other hand, regulated cell death involves a genetically-encoded molecular machinery. This machinery can be externally regulated by the inhibition of the lethal transduction signal or by improving the capacity to mount an adaptive response. The term Programmed cell death (PCD) refers to a subroutine of regulated cell death that occurs during development or, in the adult, to preserve the physiological tissue homeostasis and tune the immune response.

1.3 Immunological classification of Cell death

Cell death can be also divided in immunogenic or tolerogenic, depending on whether it induces or not an immune system response. The immunological response relies on the release of several danger signals called damage-associated molecular patterns (DAMPs). The emission of DAMPs was initially considered a feature of necrotic cell death. However, it is presently known that DAMPs are also released by apoptotic cells subjected to certain chemotherapeutic agents (e.g. Anthracycline and oxaliplatin), hypericin photodynamic therapy (PDT) or bortezomib treatment [6].

The mechanism of DAMPs emission is context and agent-dependent. DAMPs can be either released to the extracellular environment, as in the case of ATP and high mobility group protein B1 (HMGB1) or exposed on the external outer membrane, like in the case of calcireticulin (CRT) and heat shock protein 90 (HSP90). Interestingly, the ATP release by injured or stressed cells strictly relies on autophagy [7]. Once released, extracellular ATP will bind to the purinergic receptors (P2Y2 and P2X7) of macrophages and dendrocytes and function as a “find-me” signal. CRT exposure is triggered by a pre-mortem endoplasmic reticulum (ER) stress and the induction of radical species of oxygen (ROS). As for ATP releasing cells, CRT-exposing cells are recognized by CD91 positive cells (macrophages and dendrocytes) and engulfed. HMGB1, the most abundant non-histonic nuclear protein can be released either during accidental or regulated forms of cell death. Released HMGB1 interacts with Toll like receptor 4 (TLR4) of both immune and non-immune cells and triggers a local pro-inflammatory response.

1.4 Biochemical classification of cell death

The biochemical classification of cell death relies on the main molecular events associated to each cell death modality. In **Table 1**, the biochemical and structural traits of the three main modalities of cell death are summarized.

	Apoptosis	Necrosis	Autophagy
Variations/Subsets in the literature	Caspase-dependent intrinsic[a] Caspase-independent intrinsic[b] Extrinsic apoptosis by death receptors[c] Extrinsic apoptosis by dependence receptors[d]	Random or unregulated Programmed or regulated,	Macroautophagy Microautophagy Chaperone-mediated autophagy Mitophagy (but here we consider autophagy as macroautophagy)
(Biochemical) Signatures			
Mitochondrial	Caspase activation (except in [b]) Mitochondrial depolarization MOMP[a,c] Irreversible $\Delta\psi_m$ dissipation[a] CYT *c* release Release of IMS proteins[b] Respiratory chain inhibition[b] BID cleavage[c] PP2A activation[d] DAPK1 activation[d]	Loss of ultrastructure Swelling	Degradation
Cytoplasmic	Shrinkage	Swelling (including of organelles) Vacuolation Organellar disintegration	Massive vacuolization Lysosomal degradation MAP1LC3 lipidation
Nuclear	PARP cleavage Chromatin condensation DNA fragmentation (small-scale, DNA ladder)	Chromatin digestion DNA hydrolysis (smear)	SQSTM1 degradation
Structural (plasma membrane) changes	Membrane integrity preserved Formation of apoptotic bodies Membrane blebbing Phosphatidylserine externalization	Loss of integrity Blebbing Cell lysis	Double membrane-bound autophagosomes formed
Examples of trigger factors and/or conditions	Death receptor signalling[c] Dependence receptor signalling[d] DNA damage Trophic factor withdrawal Viral infections	Excitotoxicity Ischaemia Stroke Reactive oxygen/nitrogen species	Amino acid starvation Serum starvation Protein aggregates
Energy (ATP) requirement	+	–	+
(Obligatory) Caspase-dependence	+[a,c,d] –[b]	–	–
Inflammatory component	–	+	–
Major mediator(s)	Caspases (except in [b])	Calpains, CYPD, RIP-1, RIP-3 (and PARP-1 and AIF, if parthanatos is considered regulated necrosis), etc.	ATG5, ATG6 (Beclin-1), ATG7, ATG12, VPS34, AMBRA-1
Pharmacological inhibition	Caspase inhibitors, e.g. Z-VAD-fmk (except in [b])	RIP-1 inhibitors, e.g. necrostatin-1, calpain inhibitors, etc.	VPS34 inhibitors, e.g. 3-methyladenine and wortmannin
Genetic inhibition (knockout/mutation, RNAi targeting) or inhibition by protein overexpression	BCL2 overexpression[a, b] Inhibition of caspases (3, 8 and 9)[c,d] Inhibition of PP2A[d] CrmA expression[c]	Inhibition of *RIP-1* or *RIP-3*	Inhibition of *AMBRA1*, *ATG5*, *ATG7*, *ATG12* or *BECN1*

Table 1*:* Biochemical, features of apoptosis, necrosis and autophagy. Adapted from Br J Pharmacol. (2014) Apr; 171(8):2000-16

2 Apoptosis

Apoptosis derives from the Greek word ἀπόπτωσις meaning "falling off", as leaves from a tree in autumn. This term was coined by Kerr and collaborators to designate a type of cell death characterized by specific morphological traits [8]. Apoptosis is a very selective and sophisticated process involved in both physiological and pathological conditions. During embryonic and fetal development, apoptosis functions as the main mechanism of programmed cell death (PCD) for tissue sculpting and molding, canalization of ducts and tubes, formation of digits and deletion of unneeded structures [9]. After birth, apoptosis is involved in mammalian homeostatic processes. For instance, it orchestrates the clearance of approximately 60 billion of unwanted or dangerous cells per day. In the adult organism, apoptosis is also in charge of highly relevant processes such as the normal tissue turnover (skin, intestinal crypts) and the organ involution after withdrawal of trophic hormones (prostate, endometrium and mammary tissue) [10].

Evasion of apoptosis is a hallmark of cancer and an important complement to the pro-oncogene activation during cancer development [11, 12]. Genetic alterations in both intrinsic and extrinsic apoptotic pathways are common defects during tumorigenesis. For example, the B-Cell CLL/Lymphoma 2 (*BCL-2*) gene was discovered when studying a translocation between chromosome 14 and 18, a common genetic occurrence in follicular non-Hodgkin lymphoma. In general, the genetic or epigenetic deregulation of several anti-apoptotic BCL-2 family members represents a characteristic feature of numerous forms of cancers, which finally will translate into an increased resistance to chemo and radiotherapy. In the case of extrinsic pathway, the combination of epigenetic changes, down-regulation of death receptors (DR) and the overexpression of DR-related decoy receptors are commonly observed events. Nonetheless, the most usual alterations of the apoptotic machinery affect some of its upstream regulators, such as p53, class I phosphoinositol-3-kinase (PI3K)/AKT or the NF-κB pathway.

Caspase-dependent apoptosis is a type of regulated cell death (RCD) controlled by a complex cascade of events and activated by various triggers. The most distinctive trait is the activation of a family of proteases termed caspases, which are the executioners of the cell demise process.

Caspases are a family of cysteine proteases that can hydrolyze peptide bonds on the carboxyl side of aspartate residues. This fact explains the nomenclature adopted, C (cys)- Asp (Aspartate)- ases. The active site consists of a pentapeptide sequence, which contains a nucleophilic cysteine residue within a conserved motif QACXG (C, Cysteine, X can be R, Q or G) [13].

Caspases are first synthesized under the form of inactive zymogens termed pro-caspases. A pro-caspase consists of a N-terminal pro-domain (between 3 and 24 kDa), a large subunit (termed p20), which contains the caspase active site, and a C-terminal small subunit (termed p10).

Caspases can be classified on the basis of multiple parameters, for instance, the context of their activation. Based on this parameter, there are caspases that get active during apoptosis (caspase-2 -3, -6, -7, -8, -9, -10) while others are engaged during inflammation (caspase-1,-4,-5,-12L). The former classification difficults inclusion of caspase-12S and -14 in any of the previous groups. Alternatively, caspases can be classified on the basis of the position they occupy in the activation cascade. Initiator (or apical) caspases are characterized by the presence of an interaction domain (pro-domain) that allows the assembly into macromolecular activating complexes. On the other hand, executioner (or effector) caspases are placed downstream of the apical ones. As depicted in **Fig 2**, initiator caspases involved in intrinsic pathway (e.g. caspase-9) contain a caspase recruitment domain (CARD), while initiator caspases involved in the extrinsic one (e.g. caspase-8) hold a death effector domain (DED) that allows the interaction with different type of adapters (see below).

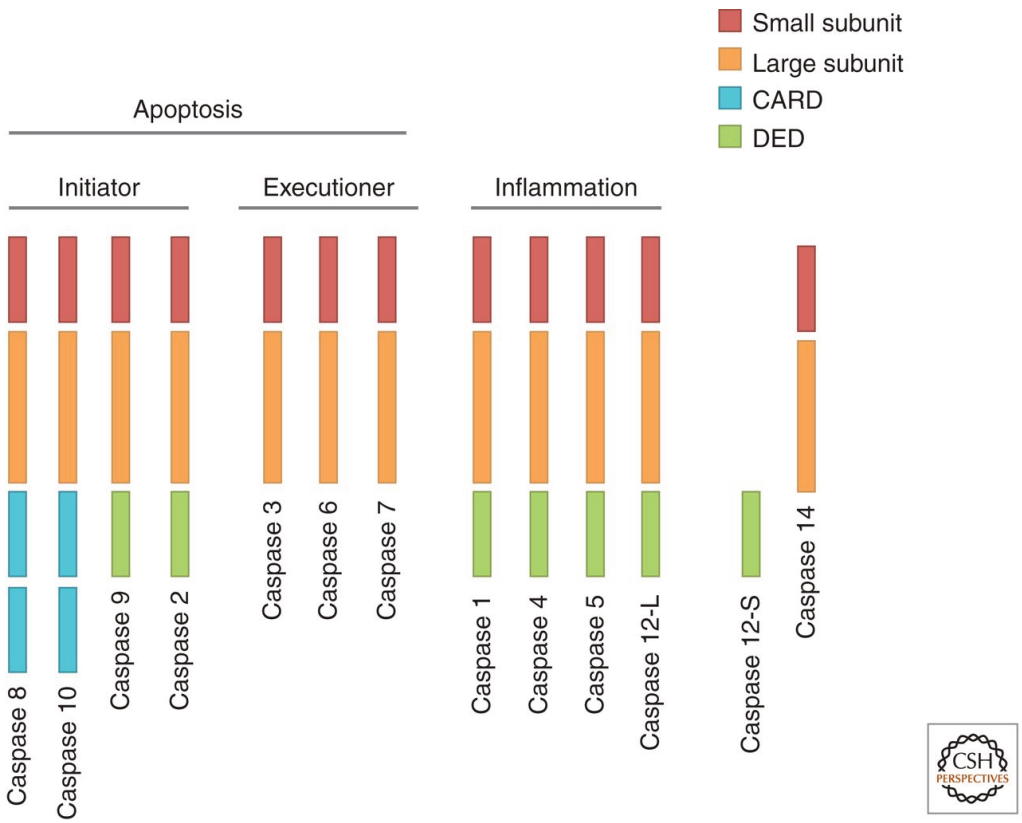

Figure 2. Domain structure and functional classification of mammalian caspases. Based on their activation, caspases can be classified into inflammatory and apoptotic. Caspases involved in apoptosis are further subdivided into initiator and executioner. Caspase 12-S and -14 are depicted in an unclassified group. CARD, caspase recruitment domain; DED, death effector domain. From Cold Spring Harb Perspect Biol. 2013 Apr 1;5(4).

Initiator pro-caspases normally exist as monomers in the cytosol. When the apoptotic stimulus is elicited, they associate and become active after dimerization within a large complex of proteins and adapters. Executioner caspases routinely exist as inactive dimers and become active in response to the cleavage by initiator caspases. This cut, which occurs between the large and the small domain, allows a conformational change and activation of the proteolytic site. The cleavage site contains aspartate residues, suggesting the possibility of an activation loop to amplify the initial cascade.

Once activated, executioner caspases-3 and -7 selectively target several hundred of substrates, while caspase-6, activated by caspase-3, cleaves a smaller group of poly-peptides. Cleavage of specific substrates by caspases contributes to the morphological and biochemical changes during apoptosis. For example, caspases target structural proteins such as filaments of actin, intermediate filaments, lamin A, lamin B, cytokeratins, On the other hand, Caspases cleave ICAD (inhibitor of caspase-activated deoxyribonuclease), a chaperone and inhibitor of the caspase-activated deoxyribonuclease (CAD). This event is central to initiate the cleavage of internucleosomal DNA, which is one of the most characteristic events to disable the functionality of a cell. In addition caspases cleave many protein kinases. This event can result in the inactivation of certain kinases or, alternatively, in the activation of others. The caspase-mediated fragmentation of protein kinase C, for instance, catalyzes the transfer of phosphatidylserine from the inner to the outer plasmatic membrane. Of note, this phenomenon is not a requirement for apoptosis to proceed and it seems to be stimulus-dependent [2]. In **Table 2**, a list of the main substrates of executioner caspases is summarized.

Substrates of Executioner Caspases	
Pathways	**Substrates**
Apoptotic regulatory proteins	BCL-2, BCL-X_L, BID, ICAD...
Cell cycle regulatory proteins	RB, p21, p27, Wee1 kinase...
Cytoskeleton proteins	Fodrin, gelsolin, actin, cytokeratin 18…
Interleukin precursor	Pro-IL-1b, Pro-IL-16, Pro-IL-18…
Proteins involved in DNA damage response	PARP, DNA-PKcs, MDM2...
Proteins involved in RNA processing	U1 70kDa, HnRNP C1 & C2...
Proteins involved in signal transduction	Calpastatin, $PhLA_2$, SRE-BP, IkB, NF-kB...
Protein kinases	PKCd, PKCt, FAK, MEKK-1…
Pro-Caspases	
Structural proteins of nuclei	Lamins A & B1, NuMA, Topoisomerase I...

Table 2: List of the main substrates of executioner caspases. The table classifies the proteolytic substrates of caspases according to their cellular function.

Caspase-dependent apoptotic cell death can occur through two well-differentiated pathways: the extrinsic and the intrinsic pathway (**Fig 3**). Both routes comprise 3 distinct phases: initiation, integration/decision and execution/degradation. The initiation stage is stimuli- and cell type-dependent. The phase of integration/decision is in charge of integrating information about internal, external pro- and anti-apoptotic cues. During this phase the "decision to die" is taken and the point of no return or commitment, is trespassed. It is during this phase that caspases, nucleases and other effector molecules become active. The execution/degradation phase is a consequence of the former phases, eventually leading to the dismantlement of the cell. In multicellular organisms, an additional stage called "clearance" occurs. Clearance refers to the engagement of the immune system to remove the apoptotic bodies.

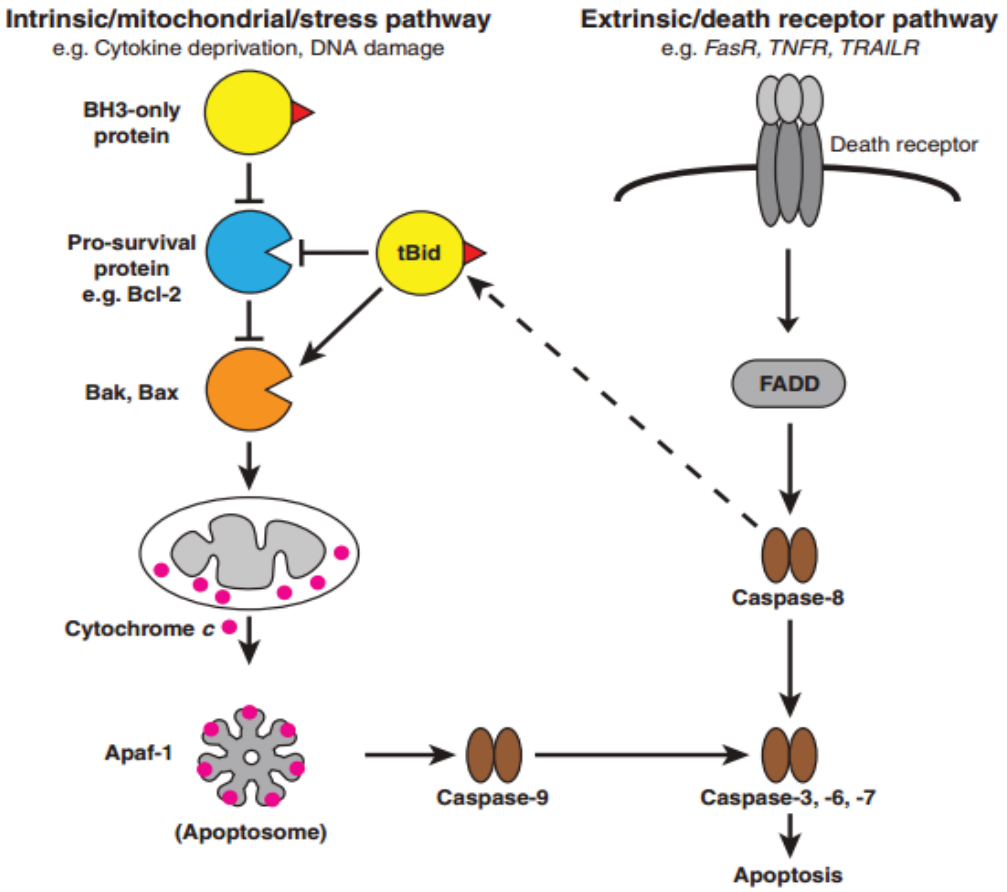

Figure 3: Apoptosis can be initiated by intracellular (intrinsic) or extracellular (extrinsic) triggers. Extrinsic or death receptor pathway is triggered by the binding of specific ligands to death receptors (FAS-R, TRAIL-R and TNF-R), which trimerize and assemble the death-inducing signaling complex (DISC). This leads to the activation of the initiator caspase, caspase-8 (or -10). In type I cells, cell death is the consequence of the direct activation of executioner caspases by caspase-8 whereas in type II, cell death requires the caspase-8-mediated cleavage of the BH3-only, BID. The new form of truncated BID (tBID) transactivates the mitochondrial apoptotic pathway necessary for the extrinsic pathway of apoptosis. The intrinsic apoptotic pathway culminates with the mitochondrial outer membrane permeabilization (MOMP) and subsequent release of cytochrome C. Cytochrome C induces the assembly of the apoptosome (comprising APAF-1, deoxy-ATP) which recruits and activates the initiator caspase-9. Caspase-9, in turn, cleaves and activates the executioner caspases (-3, -6, -7). MOMP is regulated by pro-survival and BH3-only BCL-2 family members and executed by the pro-apoptotic effectors BAX and BAK. From J Cell Biol. 2001 Dec 10;155(6):1003-15.

2.1 Extrinsinc pathway of apoptosis

Extrinsic apoptosis refers to a type of apoptotic cell death that is triggered in response to bacterial pathogens, toxins, nitric oxide, growth factors and hormones. Extrinsic apoptosis is sensed and propagated by transmembrane receptors, called Death Receptors (DRs).

DRs belong to the family of tumor necrosis factor (TNF) receptor (TNF-R), a family of type II transmembrane proteins arranged in stable homotrimers. The TNF-R family includes 29 receptors with pleotropic responses, such as proliferation, inflammation, etc. Within this family, only those owing a death domain (DD) can elicit an extrinsic apoptotic cascade.

More than 19 ligands are reported to interact with the DRs, but the only ones with a role in apoptotic cell death are tumor necrosis factor α (TNFα), the TNF-related apoptosis inducing ligand (TRAIL), FasL (also known as CD95L). Each ligand will bind to a specific death receptor, such as TNFα receptor 1 (TNFR1), TRAIL receptor (TRAIL-R) and FAS-R/CD95, respectively. Alternatively, an extrinsic apoptotic signal can also be elicited by "dependence receptors" such as the UNC-5 homolog family of receptors and deleted in colorectal cancer (DCC) family receptors, which in the absence of their ligand, netrin-1, trigger the extrinsic pathway [14].

The binding of their ligand stabilizes the homotrimers of DRs, inducing a change of conformation and allowing the assembly of a multiprotein complex termed complex IIa or "death-inducing signaling complex" (DISC). Most of the ligand-bound DRs share similar activation pathways except for TNF-R1.

2.1.1 FAS and TRAIL pathway

Upon binding to their specific ligands, FAS-R and TRAIL-R recruit the DISC, which consists of receptor-interacting protein kinase 1 (RIPK1), multiple isoforms of cellular FLICE-like inhibitory protein (c-FLIP) and a DD-containing adapter termed Fas-associated DD (FADD). Owing to the death effector domain (DED) of FADD, caspase-8 is recruited. The next step will be the dimerization and activation of these caspases.

According to the subsequent involvement or not of mitochondria, cells are classified in type I and II. In type I cells (e.g. lymphocytes) Caspase-8 cleaves and directly activates the executioner caspases. In the case of type II cells (e.g. hepatocyte and pancreatic β cell), an apical caspase transactivates the mitochondrial intrinsic pathway by cleaving BH3-interacting domain death agonist (BID). The truncated form of BID, tBID, induces then mitochondria outer membrane permeabilization (MOMP) by antagonizing the anti-apoptotic BCL-2 family members (**Fig 3**). Type I and type II cells seems to differ in the amount of inhibitors of apoptosis (IAPs) they contain [15].

The precise contribution of caspase-10 (a close of homolog of caspase-8) in the extrinsic pathway is still a debatable subject.

Notably, c-FLIP is an important regulator of this pathway. There are two variants of c-FLIP, one of 55 kDa and one of 26 kDa. Both FLIPs contain a DED domain but lack the caspase domain, acting as a dominant-negative inhibitor of Caspase-8. As a consequence TNF-α, FAS and TRAIL-induced apoptosis is dampened. Elevated expression of c-FLIP usually promotes the activation of the NF-κB pathway, triggering cell survival [16].

2.1.2 TNF-R pathway

TNF is a pro-inflammatory cytokine produced by immune cells and involved in human disease, in autoimmune disorders (e.g. Crohn's disease, rheumatoid arthritis), neurodegeneration and cancer. Two TNF receptors are reported: TNF-R1, which has a DD domain, and TNF-R2, which has not. TNF-R2 is found mostly in immune cells and endothelium. The TNF-R1 pathway is mostly involved in proliferation, anti-apoptotic and pro-survival function via its main target NF-κB. There, TNF becomes an apoptotic inducer only if NF-κB pathway is disabled. As a consequence, *in vivo*, TNF-induced apoptosis has a minor role when compared to its inflammatory signal functions.

Binding of TNFα to its receptor TNF-R1, triggers the formation of the complex I, which signals NF-κB activation. Complex I comprises TNF-R1-associated death-domain protein (TRADD), FAS-associated protein with a DD (FADD), receptor–interacting protein kinase 1 (RIPK1) also known as RIP1, TNF receptor-associated factor 2 (TRAF2) and the cellular inhibitors of apoptosis 1 (cIAP1) and cIAP2. After its recruitment, RIPK1 undergoes a K63-polyubiquitination by cIAPs. This fact allows the docking of a complex containing, transforming growth factor-β-activated kinase 1 (TAK1), TAK1 binding protein 2 (TAB2) or TAB3, which in turn attract the assembly of NF-κB kinase (IKK) complex. In an unstimulated context, NF-κB dwells in the cytoplasm and its transcriptional activity is constantly inhibited by IκBs. TNFα activates NF-kB through the ubiquitination and clearance of IκB via the activation of IKK complex, which includes IKKα and IKKβ and NEMO. IKK complex phosphorilates IκBs in two serine residues, targeting them for ubiquitination and clearance by the ubiquitin proteasome system (UPS). Unleashed, NF-κB moves to the nucleus where it exerts its pro-survival transcription activity.

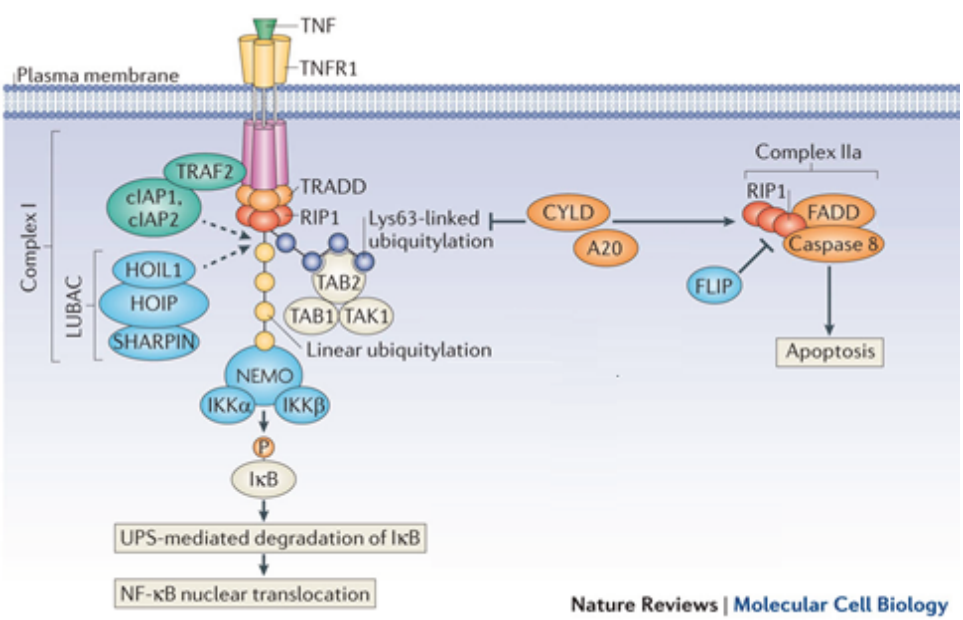

Figure 4. Schematic representation of TNF-R1 signal transduction. Binding of tumour necrosis factor (TNF) to its receptor triggers a conformational change associated to the recruitment of the membrane-associated complex I. This complex comprises TRADD (a DD-containing adapter), the death domain-containing protein kinase receptor-interacting protein 1 (RIP1) and several ubiquitin E3 ligases such as TRAF2, cIAP1 and cIAP2, and the LUBAC complex. In complex I, RIP1 is polyubiquitinylated, mediating the recruitment and activation of TGF-beta-activated kinase 1 (TAK1) and IKB kinase (IKK). Phosphorylation and subsequent UPS-mediated degradation of IKB leads to the activation of NF-κB. TAK1 is also able to phosphorylate and activate the MAPK pathway. 10-15 minutes later, deubiquitination of RIP1 and TRAF2 by CYLD or A20 brings to the assembly of complex IIa (DISC), with the subsequent caspase-8 activation and apoptosis. FLICE-inhibitory protein (FLIP) is a negative regulator of complex IIa. See text for details. Adapted from Nat Rev Mol CellBiol. 2013 Nov;14(11):727-36.

In vitro, the apoptotic activity of TNF requires translation or transcription inhibition using either cycloheximide or actinomycin D, respectively [17]. RIPK1 deubiquitination is essential for the switch of cell fate from survival to cell death. The deubiquitination of RIPK by either Cylindromatosis (CYLD) or A20 deubiquitinases leads to a molecular rearrangement of complex I into complex IIa (**Fig 4**). Deubiquitinated RIPK1 is reported to dissociate from the TNF-R1 complex, and starts the assembly of a multimeric complex comprising TRADD, FADD, pro-caspase-8 and FLICE-like inhibitory proteints (FLIPs). TRADD-dependent complex IIa induces the homodimerization and activation of pro-caspase 8, which in turn activates executioner caspases.

2.2 Mitochondrial or Intrinsic apoptotic pathway.

Mitochondria are central checkpoints in the life-death decisions of most vertebrate cells. In proliferating cells, these organelles are the powerhouse, providing the majority of ATP within a cell, through a sophisticated mechanism involving the formation of an electrochemical potential across the inner mitochondrial membrane (IMM). This gradient is produced through an electron transport chain during the transfer of electrons from NADH to Oxygen, the final acceptor, pumping protons from the matrix to the inter-membrane space. The potential energy accumulated is then turned into ATP through an endergonic reaction catalyzed by the ATP-synthase.

This delicate equilibrium can be altered by certain stresses or by pharmacological treatments. As a consequence, mitochondria stand as a central check-point in the apoptosis induction. Intrinsic apoptotic cell death is induced through the mitochondria outer membrane permeabilization (MOMP) and the release of soluble proteins (e.g. Cytochrome c), which promote the activation of caspases and the cell dismantling. The induction of MOMP dissipates the mitochondrial transmembrane potential ($\Delta\psi_m$), altering the electrochemical potential produced by the Oxidative phosphorylation (OXPHOS) process, leading to a concomitant bioenergetics crisis.

Intrinsic Apoptosis can be induced by different initiator signals, such as increased cytoplasmic Ca^{2+} concentration, ROS, hypoxia, growth factor deprivation and glucose starvation, chemotherapy etc.

The mechanism of BAX/BAK-dependent mitochondrial outer membrane permeabilization (MOMP) is a key event in intrinsic apoptosis Therefore, *Bak* $^{-/-}$/*Bax* $^{-/-}$ mice are resistant to the majority of mitochondrial apoptotic triggers. The importance of BAX and BAK during development is underscored by the high lethality (more than 90%) of double knock-out mice. The few mice able to survive to birth, show deficient apoptotic-related phenotypes [18].

The cytochrome C (cyt C) released during MOMP binds APAF-1, a scaffold protein that undergoes a conformational change and binds pro-caspase-9 via its caspase recruitment domain (CARD) [19]. The complex resulting has been called apoptosome and contains multiple subunits of APAF-1, pro-caspase-9 and deoxy-ATP. With a mechanism still controversial, the assembly of this complex activates the pro-caspase-9, which in turn activates the executioner caspase-3, -6 and -7 (**Fig 3**).

In addition, other proteins are released from mitochondria during MOMP: the IAP Mitochondria-derived activator of caspases (SMAC), also known as IAP-binding mitochondrial protein (DIABLO), the serine protease OMI (HtrA2), the apoptosis inducing factor (AIF) and endonuclease G (EndoG).

Some of them are well known triggers of caspase-independent forms of cell death. *In vitro,* caspase inhibitors, such as QVD-OPh or ZVAD-fmk, usually are only able to delay cell fate. In a context of inhibited caspases, AIF and/or Endo G can drive caspase-independent forms of death. In physiologic conditions AIF resides in the mitochondrial intermembrane space where it performs oxidoreductase functions, due to a FAD binding domain. Similarly to Cyt c, when released from mitochondria in response to apoptotic stimuli, AIF translocates to the nucleus to promote the large-scale (50 kb) DNA fragmentation. The translocation from the mitochondria to the nucleus of another endonuclease of mammalian cells called EndoG regulates the DNA fragmentation independent of caspases [20, 21].

Interestingly, the MOMP-independent release of pro-apoptotic molecules from mitochondria is still possible under specific circumstances. This is the case of those cellular perturbations that cause a sudden increase in intracellular calcium levels, ROS production, following for example acute injuries such as myocardial infarction, stroke or during ER stress. Under these settings, mitochondria show increased inner membrane

permeability due to the opening of the mitochondrial permeability transition pore (MPTP). This pore is structurally characterized by the presence of F1Fo ATP Synthase, translocator protein (TSPO) and Cyclophillin-D. In the past, ANT and Voltage Dependent Anion Channels (VDACs) were accepted proteins of the MPTP but genetic knock-down studies have ruled out their participation. The opening of the MPTP is coupled to both apoptosis and necrosis, relying on context and cell-specific cues.

2.2.1 Regulation of MOMP

2.2.1.1 Bcl-2 family members

MOMP is considered the point of no return in mitochondrial apoptosis. The integrity of the outer mitochondrial membrane is primarily controlled by proteins belonging to the BCL-2 family [18]. On a functional and structural basis, BCL-2 proteins can be classified in anti- and pro-apoptotic proteins. Within the pro-apoptotic ones, they are classically sub-divided into effectors and BH3-only proteins, though some authors prefer to refer them as group II and group III, respectively.

Anti-apoptotic Bcl-2 family members.

The anti-apoptotic proteins contain 4 conserved α-helix BCL-2 homology (BH) domains and are also referred to as BH1-BH4. Their C-terminal BH4 domain targets them to the cytoplasmic surface of mitochondria or endoplasmic reticulum. Members of this subgroup include BCL-2, BCL-X_L, BCL-W, MCL-1, A1/Bfl1, Boo/Diva, Nrf3, and BCL-B. Their anti-apoptotic effects seem to depend especially on their capacity to neutralize pro-apoptotic effectors such as BAX and BAK and interacting with BH3-only polypeptides (see below). Additionally, in some models BCL-2 can prevent apoptosis by dampening the release of Ca^{2+} from endoplasmic reticulum (ER) stores or by increasing the antioxidant capacity of cells [22].

Effector pro-apoptotic proteins: BAX and BAK

BAX and BAK represent the effector pro-apoptotic proteins. This subset of proteins is responsible of the pore formation, which eventually leads to the MOMP. Classically, they were defined by the presence of 3 BH domains, lacking the N-terminal BH4 domain. However, recent reports suggest they may also contain a BH4-like domain [23]. While BAK resides in the outer mitochondrial membrane (MOM), BAX normally localizes in the cytoplasm. In response to an apoptotic trigger, BAX C-terminal transmembrane (TM) domain loses its attachment to its hydrophobic surface groove. This elicits BAX translocation and insertion into the outer mitochondrial membrane OMM. Next, a conformational change of the N-terminus epitope of both BAX and BAK is crucial for the activation of these proteins. Following this event, the exposure of a BH3 domain seems to be driving the oligomerization of these proteins through the interaction with the hydrophobic surface groove of another activated BAK or BAX. Finally, a symmetric homodimer is formed, which in turn will oligomerise to form a large oligomeric complex [24] (**Fig 5**).

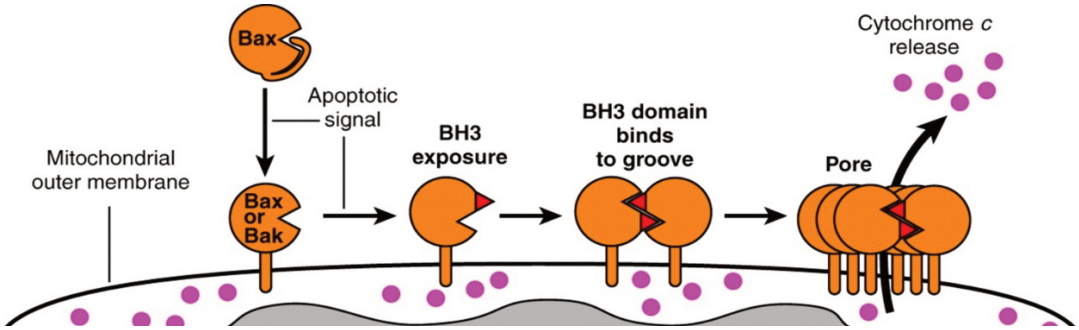

Figure 5: Model of BAK and BAX oligomerisation and pore formation. Upon apoptotic signaling, BAX is activated and translocates to the mitochondria through the eversion of its transmembrane (TM) domain. The BH3 domain (red triangle) of BAX or BAK is exposed, allowing the formation of symmetrical BAX or BAK dimers. Eventually, the dimers homo-oligomerise, forming a pore that induces MOMP and releases of pro-apoptotic factors. From J Cell Biol. 2001 Dec 10;155(6):1003-15.

Effector pro-apoptotic BCL-2 family member BAX and BAK are also reported to regulate mitochondrial dynamics. Mitochondrial dynamics, which comprises fission and fusion, is essential for maintaining mitochondrial integrity and tuning the bioenergetics status of the cell. Mitochondrial fission is regulated by dynamin-related protein 1 (Drp1), a large GTPase, which assembles in spirals around the OMM to induce its division. In its activity Drp1 is assisted by Fis1 and endophilin B (also called Bif1). Fusion is instead mediated by three large GTPase proteins: mitofusin (MFN) 1 and 2, in charge of OMM fusion and OPA-1, which mediates the internal mitochondrial membrane (IMM) fusion [25]. Monomeric BAX is for example capable of promoting mitochondrial fusion. This function is impaired during apoptosis when BAX undergoes conformational changes that prompts its insertion into mitochondrial outer membranes and oligomerization. The mechanism by means of which BAX triggers fusion is not completely elucidated but involves the interaction with mitofusion-2 (MFN2) homotypic complexes at the level of mitochondria [26].

<u>BH3-only proteins.</u>

BH3-only proteins are a heterogeneous group of pro-apoptotic polypeptides that share a restricted sequence homology of around 15-amino acids (aa) within the BH3 domain. According to their pro-apoptotic mechanism of action, they can be further divided in two sub-groups: Activators and Sensitizers/Depressors:

- Activators: BIM, tBID, PUMA are capable of directly inducing effector pro-apoptotic BAX and BAK activation, promoting their oligomerization and the cyt C release.

- Sensitizers/depressors: BAD, BIK, harakiri (HKR), NOXA, BMF. This subgroup of proteins is unable to directly activate BAX and BAK. Their pro-apoptotic function is thought to develop by two possible mechanisms: sensitization or depression. Depressors neutralize the anti-apoptotic family members while sensitizers reduced the threshold of BAX and BAX activation, without being able to trigger apoptosis by themselves.

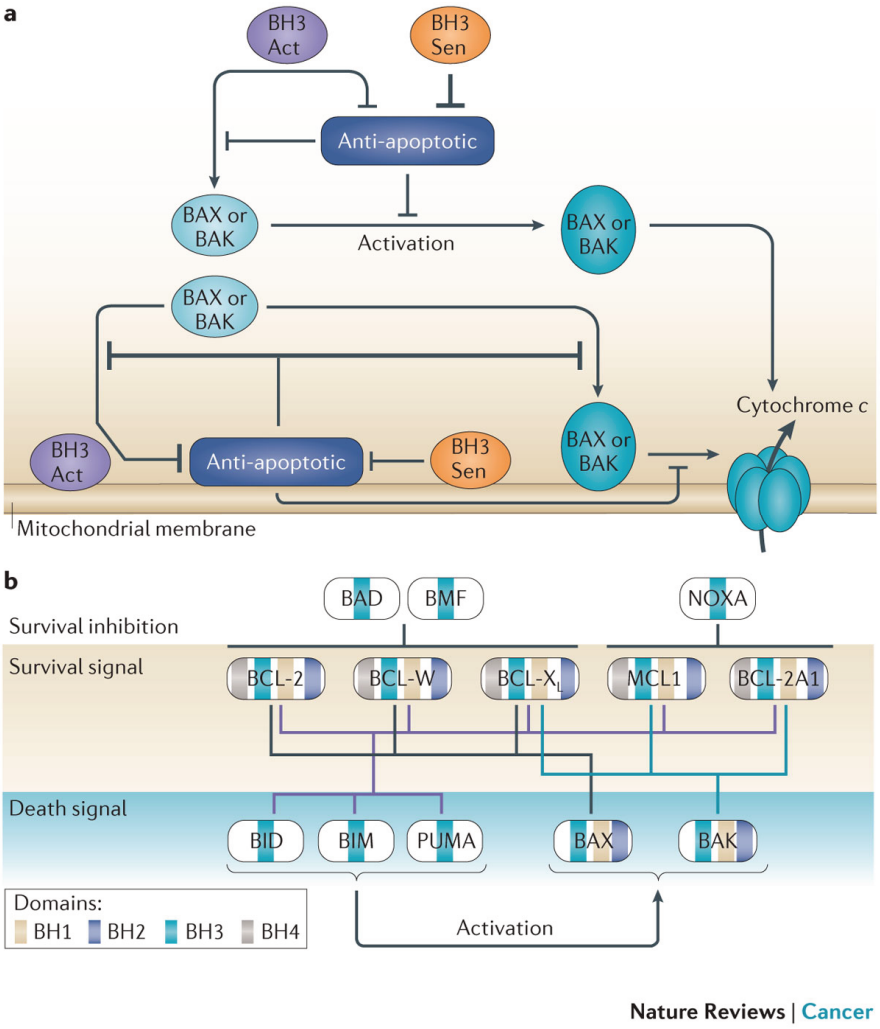

Figure 6: Regulation of BAX and BAK activation by a network of BCL-2 family members. **a)** BH3-only activators (BH3 Act) directly activate the effector pro-apoptotic proteins (BAX and BAK), thus promoting MOMP. Their activity is dampened by Anti-apoptotic BCL-2 family members. BH3 Sen (sensitizer/depressor) cannot directly activate BAX and BAK, but only prevent the interaction with anti-apoptotic proteins or in alternative reduce the threshold for the engagement of BAX and BAK. **b)** Validated interactions between BCL-2 family members are represented by connecting lines. The BCL-2 homology (BH) domain structure of each BCL-2 member is illustrated. From Nat Rev Cancer. 2013 Jul;13(7):455-65.

BH3-only proteins seem to sense and respond to different types of stress, each member sensing a specific stimulus. BID, for example, is constitutively expressed in the cell cytoplasm where it is cleaved by caspase-8, lysosomal cathepsins and other proteases. AKT, induced by many growth factors, inhibits the intrinsic pathway through the phosphorylation of BAD. Growth factors withdrawal elicit the dephosphorylation and activation of BAD followed by its release from the cytoplasmic 14-3-3 protein, which acts as a chaperone and inhibitor [27]. The activation of BIM is regulated by its release from microtubules during paclitaxel treatment. The activation of BMF responds to the release from actin filaments after loss of attachment. Finally PUMA and NOXA are synthetized upon DNA damage in a p53-dependent manner. The complex interactions between proteins of Bcl-2 family are depicted in **Fig. 6**.

2.2.1.2 P53

P53 has been termed the “cellular gatekeeper” and “the guardian of the genome”, because of its pivotal role in controlling the cell response to different stress. Adverse conditions activating p53 include for example, DNA damage, oxidative stress, virus infection, heat shock, ribonucleotide depletion and a variety of oncogenic proteins (eg. MYC, RAS, adenovirus E1A and β-catenin). By means of its transcriptional and non-transcriptional activity, p53 controls cell cycle, DNA repair, senescence, cell death and autophagy, overall functioning as a key tumor suppressor. p53 is inactivated in more than 50% of solid tumors by either direct mutation (which most often occur in the DNA binding domain) or mutation of upstream activators such as, Ataxia telangiectasia mutated (ATM) and check point kinase 1 (CHK1) or by amplification of the Mouse double minute 2 homolog gene (MDM2).[28]

p53 is structurally characterized by a N-terminus transactivation domain (TAD), which can be further subdivided in TAD1 and TAD2, a core DNA binding domain (DBD), a tetramerization domain, which is essential for p53 activity *in vivo* and a C-terminus regulatory domain.

The complex p53 regulatory network allows the modulation and expression of a specific subset of target genes, with a fine-tune response to various stresses. p53 is stabilized (and thus activated) through a series of post-translation modifications or by altering its interaction with MDM2.

Phosphorylation is the most relevant p53 modification carried out by several kinases, such as ATM, CHK1/CHK2, c-Jun N-terminal kinase (JNK) and p38. Acetylation, methylation, sumoylation and neddylation, have been also associated with the regulation of p53 and its binding to DNA.

MDM-2 is a crucial regulator of p53 content. In undamaged cells, p53 is present at low concentrations and it is usually cleared by the proteosome via its interaction with MDM2, a RING finger E3 ubiquitin ligase. The ubiquitination sites concentrate specifically at the p53 tetramerization and C-terminus domains. MDM2-p53 interaction can also be modulated by phosphorylation/acetylation of MDM2 or by the activity of ARF (also termed $p14^{ARF}$), specially under oncogenic stress. Excess activity of several oncoproteins leads to a strong induction of the tumor suppressor ARF. ARF protein arises from the alternative reading frame of *INK4A/ARF* locus and is described to interfere with MDM2-p53 interaction, thus stabilizing p53. Interestingly, *MDM2* is itself a target gene of p53, generating an auto-regulatory negative feed-back loop.

p53 is crucial in the life and death decision. According to the magnitude of an insult, it can either trigger cell cycle arrest through induction of p21 and the engagement of DNA repair mechanisms or alternatively, elicit different types of cell death (**Fig. 7**). If focusing on apoptosis, p53 elicits this form of cell death through transcriptional and non-transcriptional mechanisms.

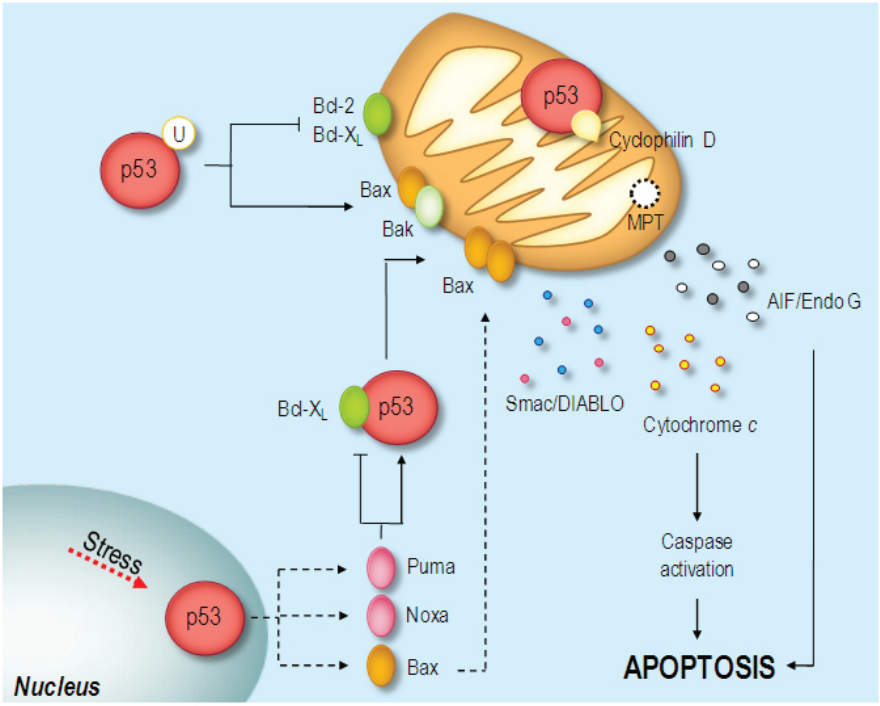

Figure 7: Role of p53 in apoptosis and necrosis. p53 can contribute to apoptosis in a transcriptional and non-transcriptional fashion. Nuclear p53 induces the transcription of pro-apoptotic BCL-2 family members, such as PUMA, NOXA and BAX. Alternatively, p53 is able to activate BAX insertion to the mitochondrial membrane. Cytoplasmic p53 is held inactive by BCL-X_L. Translocation to the mitochondria is followed by the oligomerisation of Bax, antagonizing the anti-apoptotic BCL-2 and BCL-X_L proteins. p53 can trigger necrosis through the interaction with Cyclophillin D, causing the opening of mitochondrial permeability transition pore (MPTP). From Discov Med. 2010 Feb; 9(45):145-52.

The contribution of p53 to apoptosis is mostly due to its transcriptional activity. Activated p53 moves to the nucleus where it induces the transcription of a battery of pro-apoptotic genes, for example *PUMA*, *NOXA*, *BAX*, which in turn induce or promote mitochondrial apoptosis. Data obtained from knock-out models have been decisive to prove that *PUMA* and *NOXA* are necessary for p53-mediated apoptosis. Pro-apoptotic functions of p53 can be reached through the repression of anti-apoptotic genes, such as the IAP, *survivin*.
p53 can also trigger apoptosis in a transcription-independent manner. A stress-stabilized pool of cytoplasmic p53 is the major source of p53 available for the mitochondrial translocation. Nonetheless it is still debatable how this p53 pool is generated. At the level of mitochondria, p53 can induce apoptosis by triggering the oligomerization of BAX and BAK or by dampening the anti-apoptotic BCL-2 family members, BCL-X_L and BCL-2.

2.2.1.3 IAPs

The mitochondrial pathway can still be regulated downstream of the apoptosome through the inhibitor of apoptosis (IAP) family. IAPs are an evolutionary conserved family of proteins that contains 8 members in humans (Survivin, XIAP, cIAP1, cIAP2, ML-IAP/livin, Apollon/BRUCE, NAIP and ILP-2). Members of this family are characterized by the presence of at least one copy of the BIR (baculovirus IAP repeat) domain, a zinc-binding fold that is necessary for their anti-apoptotic function. The number of BIR repeats is crucial to determine their binding partners. XIAP, one of the most well-known IAP, possesses 3 BIR domains and can target either caspase-3, -7 or -9, while Survivin, with only one copy, can only associate with caspase-9. IAPs are over-expressed in many cancers and are important for tumor cell survival since they participate into chemotherapy resistance [29]. IAPs function also as E3 ligases: cIAP1 and 2 are involved in the signal transduction of the NF-κB in extrinsic apoptosis.

3 Necrosis: Accidental versus Regulated necrosis

Necrosis derives from “nekros”, the Greek term for corpse. For two decades, necrosis has been considered only a mere accidental and unregulated cell death subroutine, caused by the action of an extremely harsh chemical, physical or mechanic insult [2]. Morphologically, necrosis has been characterized by a generalized swelling of the cytoplasm, which acquires a translucent aspect, and organelles (oncosis), as well as some atypical alterations of the DNA (condensation into small and irregular patches), the dilatation of the nuclear membrane, the rounding of the cell and eventually, the plasma membrane rupture. In addition, this subroutine of cell death can be differentiated from apoptosis by the absence of cellular fragmentation. Notably, the apoptotic corpses generated during this fragmentation will finally undergo a membrane rupture, process known as secondary necrosis and explained by the absence of phagocytic system *in vitro*.

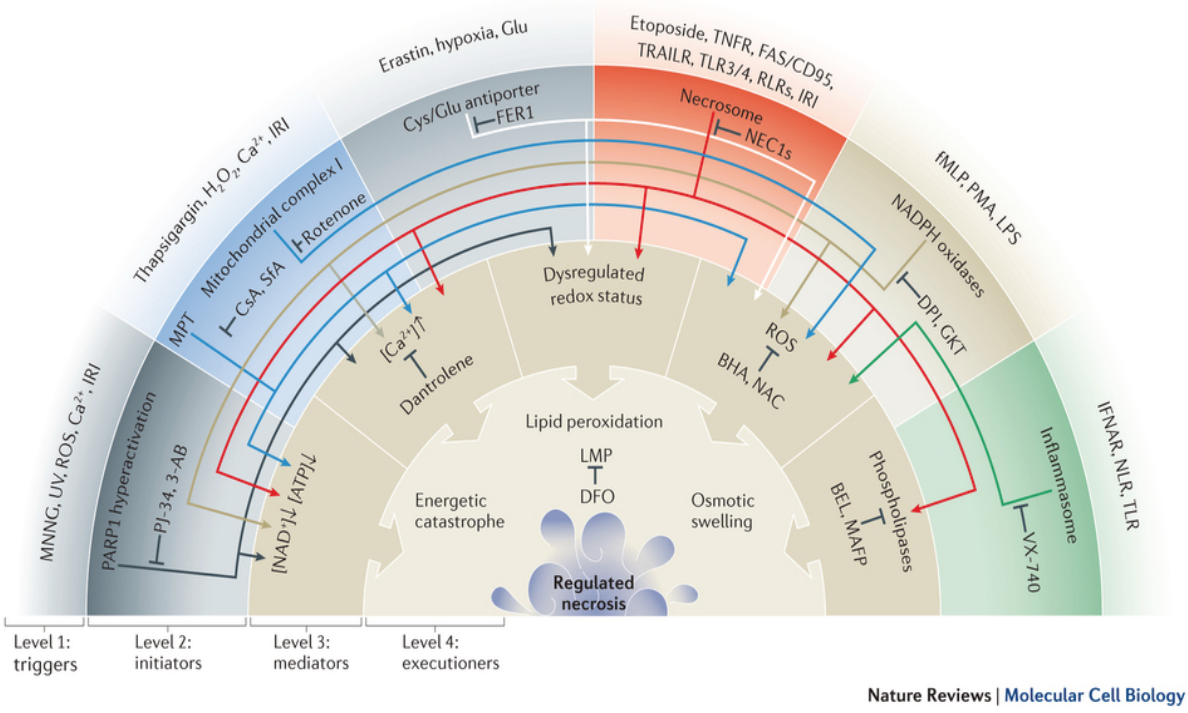

Figure 8. Schematic view of regulated-necrosis. Different types of triggers can induce regulated necrosis (level 1) by means of specific initiators (level 2). The main regulated necrosis initiators are PARP1 hyperactivation, mitochondrial permeability transition (MPT) pore, Cys/Gu antiporter, Necrosome, NADPH oxidases and inflammasome. For each initiator, colored arrows indicates the intracellular mediators (level 3). Intracellular mediators of necrosis comprise NAD^+ and ATP-depletion, Ca^{2+} overload, dysregulation of the redox status, increased production of reactive oxygen species (ROS) and the activity of phospholipases. This complex network of mediators eventually leads to the activation of necrosis executioners (level 4) leading to the energetic catastrophe, lipid peroxidation, lysosomal membrane permeabilization (LMP) and osmotic swelling. See text for details. Pharmacological inhibitors are depicted. Abbreviations: 3-AB, 3-aminobenzamide; CsA, cyclosporin A; DFO, deferoxamine; DPI, diphenylen iodonium; FER1, ferrostatin 1; fMLP, N-formylated methionyl-leucyl-Phe; GKT, GKT137831 (an NADPH oxidase 1 and NADPH-oxidase 4 inhibitor; Genkyotex); IFNAR, IFNα/β receptor; IRI, ischaemia–reperfusion injury; LPS, lipopolysaccha- ride; MNNG, N-methyl-N´-nitro-N-nitrosoguanidine; NEC1s, more specific and stable variant of necrostatin 1; NLR, NOD-like receptor; PJ-34, an inhibitor of poly(ADP-ribose) polymerase; PMA, phorbol-12-myristate-13-acetate; RIG-I, retinoic acid-inducible gene 1; RLR, SfA, sanglifehrin A; TLR, Toll-like receptor; TNFR, tumour necrosis factor receptor; TRAILR, TNF-related apoptosis-inducing ligand receptor; UV, ultraviolet light; VX-740, a caspas e 1 inhibitor. Figure adapted from Nat Rev Mol Cell Biol. 2014 Feb;15(2):135-47

After the discovery of the RIPK1/RIPK3-mediated necroptosis, the concept of regulated necrosis was introduced to define a series of necrotic cell death modalities genetically modulated, which rely on controlled biochemical events. Regulated necrosis has a prominent role in multiple physiological and pathological settings, such as ischemia reperfusion injury (IRI), diabetes, neurodegeneration, heart failure, infections and cancer [30].

Several forms of regulated necrosis have been reported: Necroptosis, Parthanatos, CYPD-mediated necrosis, Ferroptosis, NETosis, Pyroptosis. Although each subroutine is initiated by specific triggers, the cascade of biochemical mediators and executioners involved are partly shared. NAD^+ and ATP depletion, pathological ROS production, a failure in the Ca^{2+} homeostasis, activation of proteases or lysosomal membrane permeabilization (LMP), are key events in regulated necrosis. A schematic view of regulated necrosis biochemical events is depicted in **Fig 8**.

3.1 Mediators of Regulated Necrosis

3.1.1 NAD^+ and ATP depletion

Nicotinamide adenine dinucleotide (NAD) is an essential reducing equivalent that exists under two forms: the oxidized form NAD^+ and the reduced form NADH. NADH can be generated in the cytoplasm during the glycolysis and in mitochondria, as a product of the tricarboxylic acid (TCA) cycle, together with reduced Flavin adenine dinucleotide (FADH). The reducing equivalents brought by NADH and FADH enter into the electron respiratory chain and are, in turn, used by the mitochondrial oxidative phosphorylation (OXPHOS) pathway to generate ATP. NAD^+ depletion is a relevant mediator of regulated necrosis modalities and reported to occur in Parthanatos and CYPD-mediated necrosis [30]. At the cytoplasmic level, NAD^+ depletion leads to an interruption of the glycolytic flow. In an attempt to reestablish the NADH levels, ATP is used to generate NAD^+, thus depleting the internal stores of ATP. The mitochondrial NAD^+ pool withdrawal is one of the most detrimental outcomes of the MPTP opening, leading to the blockage of the OXPHOS [31].

Alternatively, a decrease of ATP can occur as consequence of the dissipation of the mitochondrial transmembrane potential ($\Delta\psi_m$), leading to the subsequent bioenergetic catastrophe. Depletion of ATP is documented in both apoptosis and necrosis. However, levels of ATP during apoptosis do not drop as abruptly as in necrosis. One explanation to this fact would be the caspase-dependent disruption of ATP-consuming processes, such as PARP-1 activity, the protein translation and the ubiquitin-proteasome system (UPS) [32]. In conclusion, the final decision to undergo necrotic or apoptotic cell death, could be determined by the bioenergetic status and the effective caspase/endonuclease activation.[33]

3.1.2 ROS

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen. ROS serve as signaling molecules to regulate physiological processes, but are also involved in different pathological conditions, such as aging, neurodegeneration, diabetes, atherosclerosis and cancer [34].

One of the main intracellular sources of ROS is the electron rich environment of mitochondrial inner membrane (IMM) during OXPHOS. Among the electron complexes, complex I and III are known to possess the highest ROS-producing capacity. On the other hand, the mitochondrial respiratory chain is a relevant target for the damaging effects of ROS [35]. Several extra-mitochondrial sources of ROS have been also documented, for instance, through the activation of a broad family of NADPH oxidases (NOXs) mainly located at the plasma membrane, through glycolysis and production of advanced glycation end products (AGEs), etc [32].

Most intracellular ROS are derived from the anion superoxide ($O_2^{\bullet -}$), which is produced by one-electron reduction of O_2. $O_2^{\bullet -}$ is converted by the mitochondrial superoxide dismutase (SOD) into H_2O_2. Both $O_2^{\bullet -}$ and H_2O_2 can then be converted into the highly reactive hydroxyl radical (OH•), by Haber-Weiss and Fenton reaction, respectively.

Excessive ROS production is scavenged by the cooperation of endogenous and exogenous as well as enzymatic and non-enzymatic, antioxidant systems. An imbalance between ROS generation and the scavenger machinery leads oxidative stress. Oxidative stress is enhanced by conditions where there is an increased electron flow through the respiratory chain (e.g. organ reperfusion after ischemic episodes) or by depletion of antioxidants (e.g. GSH). Oxidative stress ultimately leads to cell demise, that can resolve either into an apoptotic or necrotic subroutines of cell death [36].

Three are the main biological targets of ROS: proteins, nucleic acids and lipids. ROS attacks thiol groups (-SH) present in polypeptides, modifying and altering their conformation and function. Similarly, ROS is reported to affect the Ca^{2+} homeostasis, altering the Ca^{2+} channels of ER and the plasma membrane. In the same line, the oxidation of the adenine nucleotide translocase (ANT) by ROS facilitates the opening of MPTP [37].

DNA is particularly susceptible to oxidative damage. Genomic DNA damage activates a series of DNA damage response (DDR) pathways governed by master regulators, such as p53 or PARP-1, which in turn participate into the commitment into cell death or survival.

Another important target of ROS oxidative activity are the polyunsaturated fatty acids (PUFAs) present in cellular membranes. Lipid peroxidation of PUFAs leads to the formation of reactive aldehydes (e.g. 4-hydroxynonenal), which can destabilize the integrity of both plasma and organelles membranes. In addition, lipid peroxidation and plasma membrane disruption can be the consequence of cytosolic phospholipase A_2 and Lipoxygenase (LOX) activation [38].

3.1.3 Calcium

Ca^{2+} is an important signal mediator controlling different cellular processes such as gene transcription, muscle contraction and cell proliferation. Viable cells maintain the calcium gradient across plasma membrane mainly through the Na^{+}/Ca^{2+} exchanger *(*NCX) and the plasmatic Ca^{2+} pump (PMCA), also called Ca^{2+} ATPase. As a consequence of this ATP-dependent process, extracellular calcium concentration is ~10 folds higher than in the intracellular compartments. Most of intracellular calcium is stored in the ER by means of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA). It is released back to the cytoplasm through ryanodine (RyR) and inositol-1,4,5-trisphosphate receptors ($Ins(1,4,5)P_3R$). Like many other mediators, cytosolic calcium spikes may trigger either apoptosis or necrosis, and the specific cell fate subroutine engaged seems to depend on the severity of the concentration increase.[39]

Failure of Ca^{2+} homeostasis in necrosis has two main consequences: Ca^{2+} mitochondrial overload and activation of Ca^{2+}-dependent proteases. Ca^{2+} mitochondrial overload is responsible of the stimulation of the TCA cycle with a subsequent enhancement of electron flow leading to ROS overproduction. This effect is due to the Ca^{2+}-mediated positive regulation of enzymes such pyruvate dehydrogenase, NAD^{+}-dependent isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase. At the mitochondria level, in addition, Ca^{2+} boost is described to induce the opening of the MPTP. On the other hand, the increase of Calcium activates Ca^{2+}-dependent proteases, especially calpains, which cleave and inactivate NCX. This process will be sustaining an intracellular Ca^{2+} increase and, finally, the destabilization of the lysosomal membrane leading to the lysosomal membrane permeabilization (LMP) (see below).

3.1.4 Lysosome and LMP

Under physiological conditions, lysosomes are fundamental organelles for degradation of a plethora of molecules and biological structures. The lysosome has also emerged as a significant hub of the cell death machinery. LMP and the subsequent extra-lysosomal release of hydrolases are reported events in both apoptosis and necrosis initiated in extra-lysosomal sites. However, the lysosome, its hydrolases and its elevated oxidative potential are enabling the capacity of the lysosome to act as a cell death initiator organelle.

Lysosomal membrane integrity is preserved through the action of a series of cytoprotective factors. Serpins, a family of protease inhibitors, are able to block LMP through the inhibition of cathepsins. Similarly, another relevant factor is HSP70. HSP70 localization and association to the lysosomes makes them less prone to LMP. In the same line, HSP70 is reported to delay TNF, heat shock and oxidative stress-driven necrotic cell death.[32] LMP occurs as a response to a variety of stimuli such as ROS-dependent membrane permeabilization or the activation of two lysosomal lipases, the acid sphingomyelinase (A-SMase) or acid ceramidase (A-CDase). The lysosomal enzyme A-SMase converts the membrane lipid sphingomyelin into ceramide, which in turn can be further converted into sphingosine by the A-CDase. While ceramide has shown to block mitochondrial respiratory chain complex III, the lysosomotropic sphingosine, can permeabilize the cell membrane through its detergent-like properties [40].

The intraluminal lysosomal compartment is rich in H_2O_2, reduced iron ions (Fe^{2+}) and reducing equivalents, being the perfect microenvironment for the Fenton reaction. It contains desferrioxamine, an iron chelator, which is reported to prevent the lipid peroxidation and the LMP induced by oxidative stress [32].

3.2 Subroutines of Regulated necrosis

3.2.1 Necroptosis

Necroptosis is a necrotic cell death modality initially described in L929 cells treated with TNF-α after pharmacologically inhibiting caspases. The NCCD 2015 defines Necroptosis as a RCD subroutine that critically depends on the kinase activity of RIPK1 -inhibited pharmacologically by necrostatin-1-, RIPK3 and on the execution by mixed lineage kinase domain-like (MLKL) -inhibited by necrosulfonamide- [2].

Death receptor-mediated apoptosis and necroptosis are interconnected subroutines of cell death but mutually exclusive. RIPK1 plays an important role in both the transduction of apoptotic extrinsic pathway and also, in necroptosis.

Necroptosis can be triggered by several insults, including alkylating DNA damage, exitotoxins and ligand DRs (TNFα, FasL and TRAIL), but also by anticancer drugs such Shikonin and GX15-070 (Obatoclax). *In vivo*, necroptosis has been reported to occur in the absence of caspase inhibition during myocardial infarction, ischemic or traumatic brain injury, renal ischemia-reperfusion injury (IRI) but also during the systemic inflammatory syndrome in the liver and immune cells [30].

The induction of necroptosis shares several steps with the TNF-α signaling pathway (**Fig 9**). TNF-α transduces a plethora of different signals: canonical and non-canonical NF-κB activation (complex I) and DISC-mediated caspase-8 activation (complex IIa). In the context of an inhibited caspase-8 and after TNF-α exposition, RIPK1 docks a microfilament-like complex termed necrosome, which comprises RIPK1, RIPK3, FADD and TRADD.

Necroptosis can be activated also in absence of TNF-α with the formation of a TRADD-independent complex termed Ripoptosome (also called complex IIb), which comprises RIPK1, RIPK3, FADD and FLIP-$_L$/-caspase-8 heterodimers. Ripoptosome assemble occurs under genotixic stress and critically depends on the autodegradation of IAPs, a process enhanced by the release of SMAC/DIABLO. Ripoptosome assembly stimulates caspase-8 activation and/or necroptosis, depending on cellular context.

Caspase-8 and FLIP-$_L$ are negative regulators of necrosome and ripoptosome assemblies. Active caspase-8 and the heterodimer caspase-8/FLIP-$_L$ are reported to cleave RIPK1 and RIPK3, as well as CYLD, preventing RIP1K and RIPK3 activation. Interestingly, some specific form of necroptosis RIKP1-independent but RIPK3-dependents has been reported [41].

In order to get active RIPK1 and RIPK3 undergo reciprocal auto- and trans-phosphorilation. The phosphorylation of RIPK3 at Ser227 and 232 is essential for the subsequent recruitment of Mixed lineage kinase domain like (MLKL), the final executioner of necroptosis. MLKL contains a N-terminus four α-helix bundle (the functional domain of MLKL) connected to the C-terminus kinase-like domain by two α-helix linker. In normal conditions, MLKL dwells in the cytoplasm as a monomer. When RIPK3 is engaged and activated within the necrosome, it induces the phosphorylation of MLKL at Thr357 and Ser358 sites, which destabilizes MLKL, allowing its homo-oligomerization and subsequent binding to phosphatidylinositol phosphate lipids (PIPs) and the mitochondria-specific phospholipid, cardiolipin (CL). The interaction of MLKL with PIP- or CL-enriched membranes is indispensable for MLKL-dependent necroptosis.

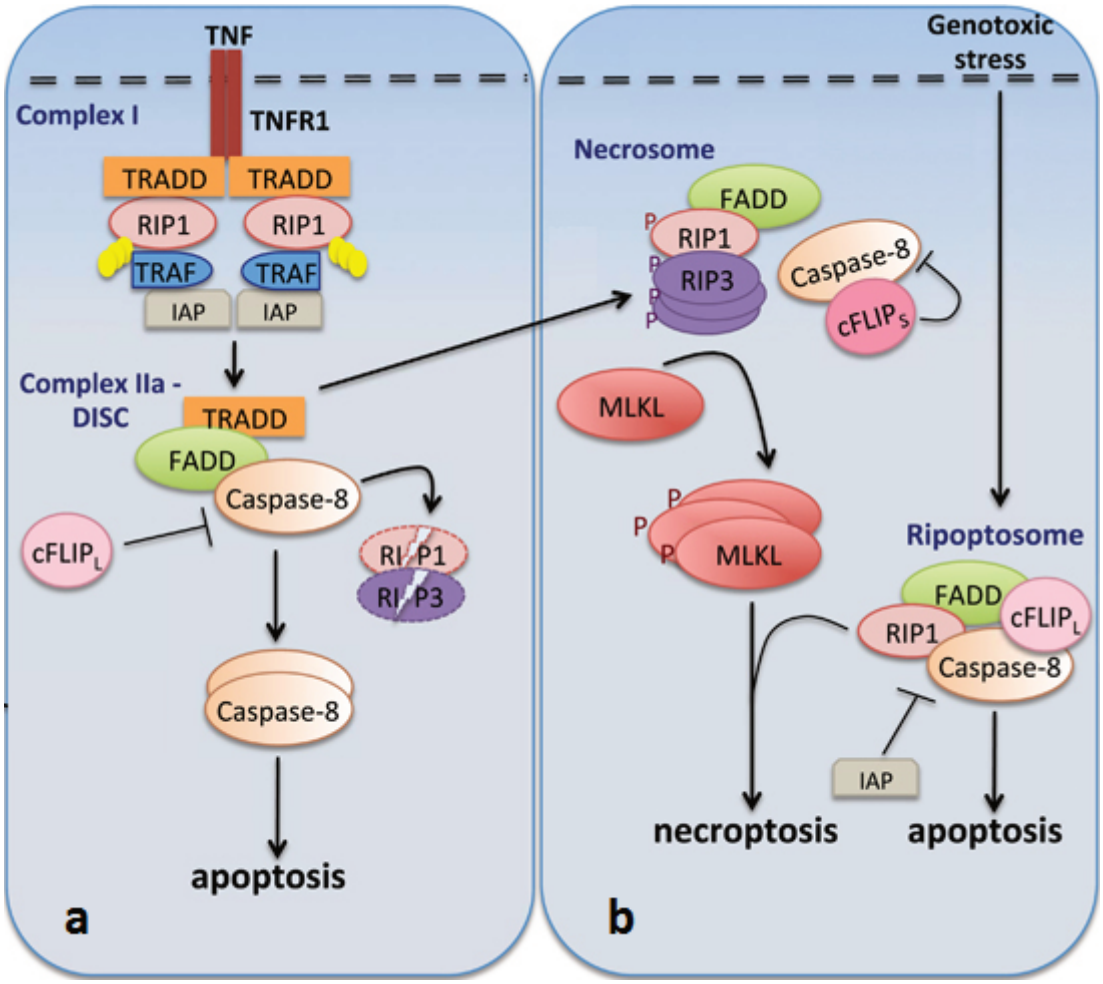

Figure 9: Necroptosis signaling pathway. **a)** CYLD- or A20-dependent deubiquitination of RIP1 allows its dissociation from complex I and the assembly of complex IIa (DISC). Complex IIa is a requisite for caspase-8 activation and the induction of extrinsic apoptosis. Activated caspase-8 cleaves RIP1 and RIP3 blocking necroptosis. **b)** When caspase-8 is inhibited, RIP1 and RIP3 aggregate under the form of a micro-filament like complex (Necrosome), triggering MLKL-dependent necroptosis. In cells subjected to a genotoxic stress or IAPs depletion, the ripoptosome complex is assembled in a TNF-independent manner. According to the presence or absence of active caspase-8, ripoptosome can trigger either apoptotic or necroptotic cell death. Adapted from Cell Death Differ. 2015 Apr;22(4):526-39.

MLKL translocates to the plasma membrane via its interaction with PI(4)P and PI(4,5)P2. At this level, MLKL is reported to induce an intracellular Ca^{2+} spike through activation of TRPM7 (a non-voltage-sensitive ion channel) and Na^{2+} influx, which potentially contribute to the necrotic phenotype. During necrosis, CL, which normally resides in the inner membrane is exposed to MLKL. MLKL is reported to trigger mitochondria-dependent ROS generation and mitochondrial fusion, the latter through the activation of PGAM5 phosphatase, which dephosphorilates and inactivates Drp1, a dynamin-related GTPase necessary for mitochondrial fission.

3.2.2 Parthanatos

Parthanatos is a form of cell death characterized by the overactivation of poly(ADP-ribose) polymerase 1(PARP-1) and the PAR-dependent release of AIF from mitochondria. The term parthanatos was coined to describe this death modality, combining the word PAR and *Thanatos*, the personification of Death in Greek mythology.

The uniqueness of parthanatos makes its definition controversial since some authors define it as a form of caspase-independent cell death, while others a form of regulated necrosis. Indeed, parthanatos morphological traits are partly overlapping between apoptosis and necrosis. Morphologically, parthanatos is characterized by plasma membrane rupture, large scale DNA fragmentation (50 kDa), while organelles swelling is not reported [42].

In physiological conditions, PARP-1 is involved in the preservation of genomic stability and it is part of the DNA damage response (DDR). It mainly functions as a sensor of single-strand DNA breaks (SSB). When a DNA SSB occurs, PARP-1 binds to the damaged DNA and its activation leads to a NAD-dependent PARylation of chromatin proteins surrounding the DNA breaks, which in turn signals and activates multiple repair enzymes such as DNA topoisomerases, helicases and ligases.

Parthanatos is triggered by several DNA damage triggers such as alkylating agents (MNNG), ROS, increase of Ca^{2+} concentration. In situation of profound DNA damage, hyper production of PAR causes cell demise. The mechanism involved it is not completely clarified, but involves the decrease of NAD^+ and ATP subsequent to PARP-1 enzymatic hyperactivity and the interaction of PAR with AIF. PAR induces AIF release but also activates calpains, which in turn, generate truncated AIF (tAIF). tAIF, through a non-specified endonuclease, cleaves DNA engaging a positive feedback loop for PARP-1 activation [42].

3.2.3 CYPD-dependent regulated necrosis

The aperture of MPT pore can lead to either apoptosis or regulated necrosis [36]. As described before, MPTP is a dynamic complex whose composition has not completely been addressed. CYPD is a prominent regulator of MPTP, which is inhibited by cyclosporin A (CsA). CYPD-dependent regulated necrosis, is triggered by IRI, stroke, myocardial infarction, ROS, thapsigargin, and Ca^{2+} boost. The necrotic cell death due to the opening of the MPTP results from the loss of mitochondrial potential and subsequent decrease of ATP and release of mitochondrial NAD^+, which in turn causes glycolysis inhibition.

More recently, p53 has been reported as a regulator of CYPD-mediated necrosis in ischemic tissues. Upon H_2O_2 treatment, MEFs *Bax*$^{-/-}$/*Bak*$^{-/-}$ (DKO) and WTs undergo a necrotic cell death characterized by p53 mitochondrial accumulation and its interaction with CYPD. Genetic ablation of p53 or treatment with CsA is able to rescue both DKO and WT MEFs cell from H_2O_2-induced necrotic cell death [43].

3.2.4 Ferroptosis and Oxytosis

Ferroptosis and oxytosis remain two controversial cell death modalities. Considered by some authors as a form of regulated necrosis, others hold them as unique subroutine entities.

Ferroptosis and Oxytosis both depend on the Xc–Cys/Glu antiporter and a subsequent decrease of Glutathione (GSH). In physiological settings, Xc–Cys/Glu antiporter is responsible of the exchange between L-Cys and intracellular L-Glu across the plasma membrane, which is necessary for GSH synthesis.

Ferroptosis was firstly described in RAS-transformed tumor cells lines treated with erastin, an inhibitor of Xc–Cys/Glu antiporter [44]. Fenton-like reactions and NOX-1-dependent ROS generation are the main drivers of ferroptosis, which is in addition strictly depends on iron. In this setting, GSH depletion leads to the loss of function of GSH peroxidase 4 (GPX4) with a ROS increase and the subsequent lipid peroxidation.

In the case of oxytosis, the depletion of GSH activates 12 and 15-lypoxygenase (LOX12, LOX 15), which in turn, mediate mitochondrial-dependent ROS production and increase of cGMP. cGMP opens a cGMP-gate channel with an increase of Ca^{2+}, activation of calpains and LMP.

3.2.5 NETosis

NEtosis is a form of regulated necrosis typically observed in neutrophils as an anti-bacterial immune response. This cell death modality is characterized by the release of neutrophil chromatin, thus constructing adhesive and deadly extracellular traps (NETs) for bacteria. NOX-1 activation, which can contribute also to other necrotic modalities, is the main molecular event in the execution of NETosis.

3.2.6 Pyroptosis

The term Pyroptosis was introduced to describe a cell death subroutine engaged by macrophage infected with *Salmonella enterica*.[45] Pyroptosis is part of the antibacterial innate immune defense and it is characterized by rapid release of cytosolic contents following the caspase-1-dependent formation of plasmatic pores. Several initiators of pyroptosis have been reported, including toll-like receptors (TLRs), NOD-like receptors (NLRs), exposure of microbial pathogen-associated molecular pattern (PAMPs), endogenous damage-associated molecular pattern (DAMPs), ultraviolet B (UVB) irradiatiaons.[30] These triggers induce the assembly of the inflammasome, which comprises caspase-1 (whose activation requires in some settings caspase-11), NLR family, pyrin domain containing 1 (NLRP1), NLRP3 and apoptosis associated speck-like protein containing a CARD domain (ASC). Caspase-1 induces the activation and release of its two main targets, IL-1β and IL-18 and the subsequent amplification of the inflammatory damage.

4 Autophagic cell death (ACD)

The most updated definition of "autophagic cell death" by the NCCD claims that is a cell death subroutine limited or delayed by pharmacologic or genetic interventions targeting at least two distinct components of the molecular machinery of autophagy [2] and accompanied by the presence of an active autophagic flux.

Studies in model organisms, such as *Dictyostelium Discoideum*, *C. elegans* and *Drosophila melanogaster*, have provided multiple evidences regarding the participation of autophagic cell death in the development of these organisms. However, no clear prove exists of its role in mammalian embryogenesis until perinatal stages [46].

The autophagy-driven cell death is integrated in a cell death network where autophagy activity is intertwined with other cell death modalities. This might add a level of complication to the studies of autophagic cell death during the development of the aforementioned organisms. For instance, the single disruption of apoptosis or autophagy in *C. elegans* has no lethal effect for a normal development and does not result in early embryonic lethality [47], probably owing to compensatory mechanisms. On the contrary, the disruption of both processes unables these compensatory pathways and triggers massive cell death.

A unique model to address if autophagy is indeed a cell death or survival mechanism comes from the amoeba *Dictyostelium Discoideum*, which lacks from the apoptotic machinery. In the presence of abundant nutrients, *D. Discoideum* exists in a vegetative state. Upon starvation, the amoebas aggregate in a multicellular fruiting body, in a process that requires PCD. While autophagy is normally engaged upon starvation, a second signal, the differentiation inducing factor-1 (DIF-1) is required for cells to undergo PCD by autophagic cell death [48].

Drosophila melanogaster is another model organism to examine the role of autophagic cell death in a developmental context. During the *Drosophila* life cycle from larval to adult stage, obsoleted tissues, such as midgut and salivary glands are removed through PCD. While the removal of salivary glands seem to rely on parallel activity of apoptosis and autophagy, apoptosis seems dispensable in the case of midgut regression. Interestingly, the components of the autophagic pathway involved in PCD seem to not be completely overlapping with the ones required for starvation-induced autophagy [49].

Putting aside the development models, autophagy-driven cell death has been reported in mammalian adult cells cultures. In most cases, inactivation of apoptosis was a requisite to observe autophagic cell death. This is the case of double knockout *Bax*$^{-/-}$/*Bak* $^{-/-}$ MEFs, that subjected to long-term treatment with etoposide or staurosporine, undergo 3-MA-sensitive and Beclin-1 and *Atg5*-dependent cell death [50]. Similarly, cell death by caspase-8 inhibition in mouse L929 cells was suppressed knocking-down *Beclin-1* or *Atg7*. Further investigations proved that the specific degradation of the anti-oxidant catalase into the autophagolysosomes and the ensuing accumulation of ROS were at the cause of the observed caspase-independent cell death [51].

Alternatively, in other models, the suppression of caspase activity is not necessary. Forced expression of H-RAS in human ovarian surface epithelial (HOSE) cells causes a caspase-independent cell death and massive induction of autophagy. RAS-induced death relies on the induction of NOXA and Beclin-1. In the presence of NOXA, Beclin-1 dissociates from MCL-1, resulting in the promotion of autophagy. The genetic ablation of *ATG5* and *ATG7, Beclin-1* and *NOXA* is able to rescue from cell death [52].

Basal levels of caspase-10 activity regulate autophagy of myeloma cell lines, through the proteolysis of BCLAF1. When caspase-10 is inhibited, BCLAF1 is stabilized and displaces BCL-2 from Beclin-1, thus inducing autophagy. The inhibition of caspase-10 leads to a form of autophagic cell death, which is abrogated

by the genetic ablation of components of the autophagy machinery (*ATG5* and *Beclin-1*) and the knockdown of *BCLAF1* [53].

Autosis represents a recently described autophagic cell death paradigm. Curiously, autosis occurs in cultures treated with autophagy-promoting peptides and *in vivo* models of ischemia-reperfusion. Beclin-1-derived peptide (Tat-Beclin-1) is a strong promoter of autosis, which is blocked by either the pharmacologic or genetic inhibition of autophagy. Ultrastructurally, Tat-Beclin-1 cell death is characterized by rapid shrinkage of the nucleus with a portion of its surface becoming concave, followed by the focal membrane rupture. By means of a 5000 compounds screening, the plasma membrane Na^{2+}/K^{+}-ATPase was identified as a key player in autosis. Another unique feature of autosis is represented by an increased substrate adherence of dying cells which could be explained by the role of Na^{2+}/K^{+}-ATPase in cell-adhesion [54]. *In vitro*, autosis could be engaged for example, by the subpopulation with the highest level of autophagy, which therefore become substrate-adherent during nutrient starvation [55]. The authors reported also a retroactive form of autosis triggered by another autophagy-inducing peptide, Tat-Flip α2, which acts by releasing ATG3 from c-FLIP [56].

5 Homeostatic Quality control mechanisms

Eukaryotic cells possess specific mechanisms for quality control of proteins and organelles. Globally, these mechanisms are part of an internal homeostat. The molecular chaperones are the frontline machinery in charge of protein folding/refolding and sequestration of abnormal proteins, while the ubiquitin protein system (UPS) and the lysosomal-autophagy system are the two main intracellular degradative machineries.

5.1 Molecular chaperones

Conditions of inefficient or increased translation as well as expression of mutant forms of proteins are the main causes of unfolded proteins accumulation [57]. Cancer cells synthetize a large amount of proteins to cope with their high proliferative and metabolic rate and are thus, extremely prone to proteotoxicity. As a consequence, elevated expression of individual heat shock proteins (HSPs) and the heat shock transcription factor 1 (HSF-1), have been reported in a wide array of human cancers, leading to the concept of non-oncogenic cancer addiction [58]. Accordingly, HSF1 and HSP70 are reported to be necessary for the survival and growth of several types of cancer cells, but dispensable for normal ones.

Protein misfolding is sensed by compartment-specific stress response pathways in order to preserve proteohomestasis. These mechanisms include for example, the ER unfolded protein response (UPR^{ER}) and the cytoplasmic heat-shock response (HSR) [59].

UPR^{ER} responds to an accumulation of unfolded protein in the ER. ER stress occurs when the unfolded proteins rate formation exceeds the buffering capacity of the ER folding machinery. ER stress activates three signaling pathways: inositol-requiring protein 1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6*)*. The UPR^{ER} is triggered by the direct interaction with unfolded proteins or the stress-dependent displacement of BiP/GRP78 (an HSP70 family member protein). The three activated branches have direct or indirect transcriptional effects, which determine upregulation of chaperones, increased efficiency of membrane trafficking pathways, induction of autophagy and genes involved in protein degradation, such as the ER-associated protein degradation (ERAD). During prolonged or severe ER stress, the upregulation of apoptotic genes, like for instance CHOP, triggers apoptotic cell death.

The HSR response is rapidly induced by stresses, such as heat shock. HSF-1 is the master regulator of HSR and is maintained in an inactivated state by the interaction with HSP90, HSP70 and the cofactor HSP40. With the appearance of misfolded proteins, HSF-1 dissociates from HSPs, forming a homotrimer that translocates to the nucleus where it induces the transcription of chaperones, proteasome subunits and proteins that facilitate trafficking genes [60].

HSC70/HSP70 and HSP90 molecular chaperones are the two major cytosolic folding machinery responsible of HSR. In the cytoplasm, chaperone-mediated proteostasis involves several strategies: re-folding, degradation or the sequestration of the misfolded proteins [57] (**Fig. 10**).

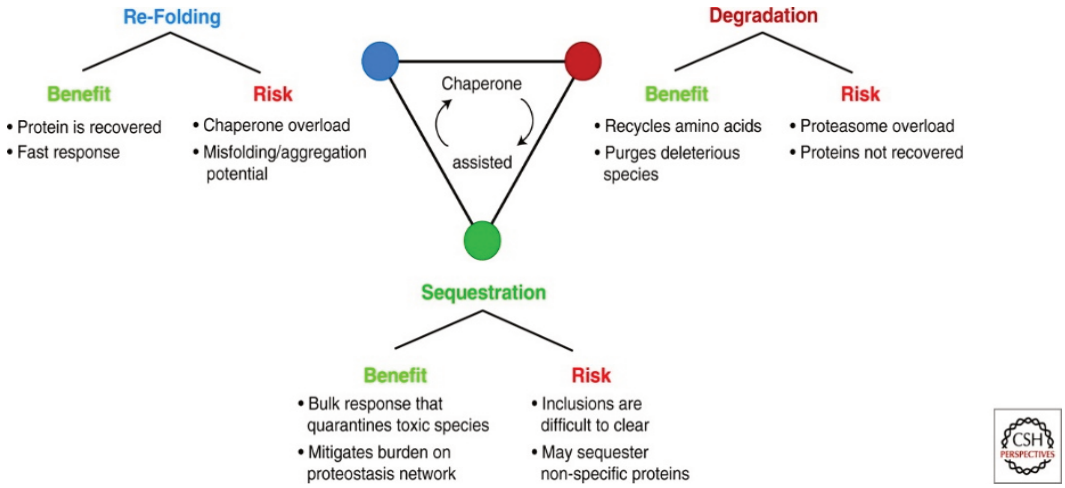

Figure 10: Strategies of protein homeostasis. In a process assisted by molecular chaperones, misfolded proteins can be refolded, degraded, or sequestered. Risks and benefits of each strategy are illustrated. Cold Spring Harb Perspect Biol. 2011 Aug 1;3(8):a004374.

HSC70 is a protein constitutively expressed at low level in viable cells, functioning as a basal housekeeping chaperone. HSP70 is the main stress-inducible chaperone and it constitutes a central hub within the chaperone system. Finally, the remaining members of the family are involved in sensing misfolded proteins in specific compartments, like for example HSP70-5 (also known as BiP or Grp78) in the ER and HSP70-9 in the mitochondrion [61].

In eukaryotes, HSP90 activity is essential in unstressed condition. HSP90 is required for assisting in the folding of a wide number of client proteins. More than 200 proteins are HSP90 "clients", a list that includes protein kinases, transcription factors (e.g. p53) and steroid hormone receptors (SHRs) [62].

HSP70 and HSP90 are induced upon accumulation of misfolded proteins in the cytoplasm. HSP70/HSP90-driven folding of polypeptides is an ATP-dependent mechanism. Some client proteins are directly folded by HSP70, while in other cases, HSP70 acts like an obligate HSP90 co-chaperone. Both HSP70 and HSP90 exert their refolding activity undergoing cycles of ATP/ADP conformational changes. Co-factors like HSP40, regulate the HSP70 activity through the control of the ATP hydrolysis step. The mechanism of refolding requires cycles of chaperone-bound and –free states, until the misfolded polypeptide has reached the native conformation. (**Fig. 11**)

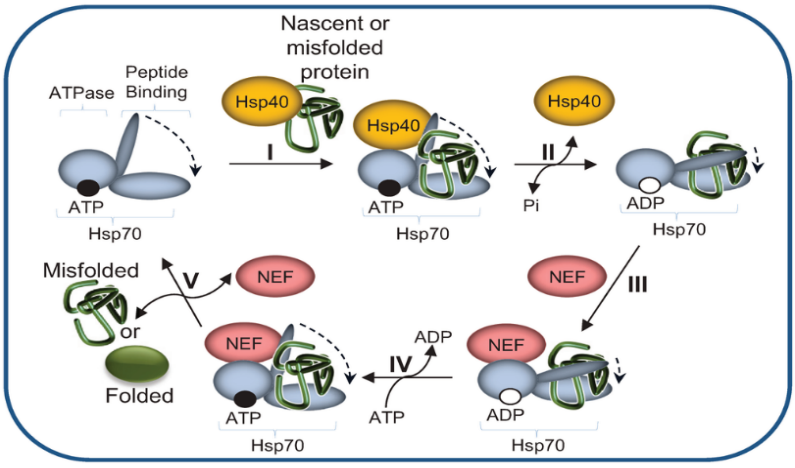

Figure 11: The Hsp70 cycle of refolding. **(I)** Hsp40 mediates the delivery of misfolded (or nascent) protein to ATP-bound Hsp70; **(II)** Hsp70 conformational change upon hydrolysis of ATP to ADP. **(III-IV)** NEF (Nucleotide exchange factor) binds to Hsp70, catalyzing the dissociation of ADP. **(V)** ATP binds to the Hsp70's ATPase domain, enabling substrate release. Extrapolated from Biomolecules. 2014 Jul 17;4(3):704-24.

If misfolded protein damage is beyond repair, the chaperone network directs them to a degradative pathway. Alternatively, misfolded proteins can be sequestered in detergent-insoluble structures, termed aggregosomes. The two main degradative pathways for misfolded proteins are ubiquitin-proteosome system and chaperone mediated autophagy (CMA). The proteins within an aggresome are normally ubiquitinated and enclosed by intermediate filaments (IF) proteins. Aggregosomes can be finally cleared by a selective form of macroautophagy, termed aggrephagy [63].

5.2 The Ubiquitin/Proteasome system (UPS)

UPS is the major extra-lysosomal degradative machinery responsible of targeting nuclear, cytoplasmic and ER short-living proteins. Proteins are marked for UPS through the binding of long ubiquitine tails, which guide the tagged proteins to the 26S proteasome, a highly conserved multi-catalytic ATP-dependent protease complex. The main components of UPS are depicted in **Fig 12**.

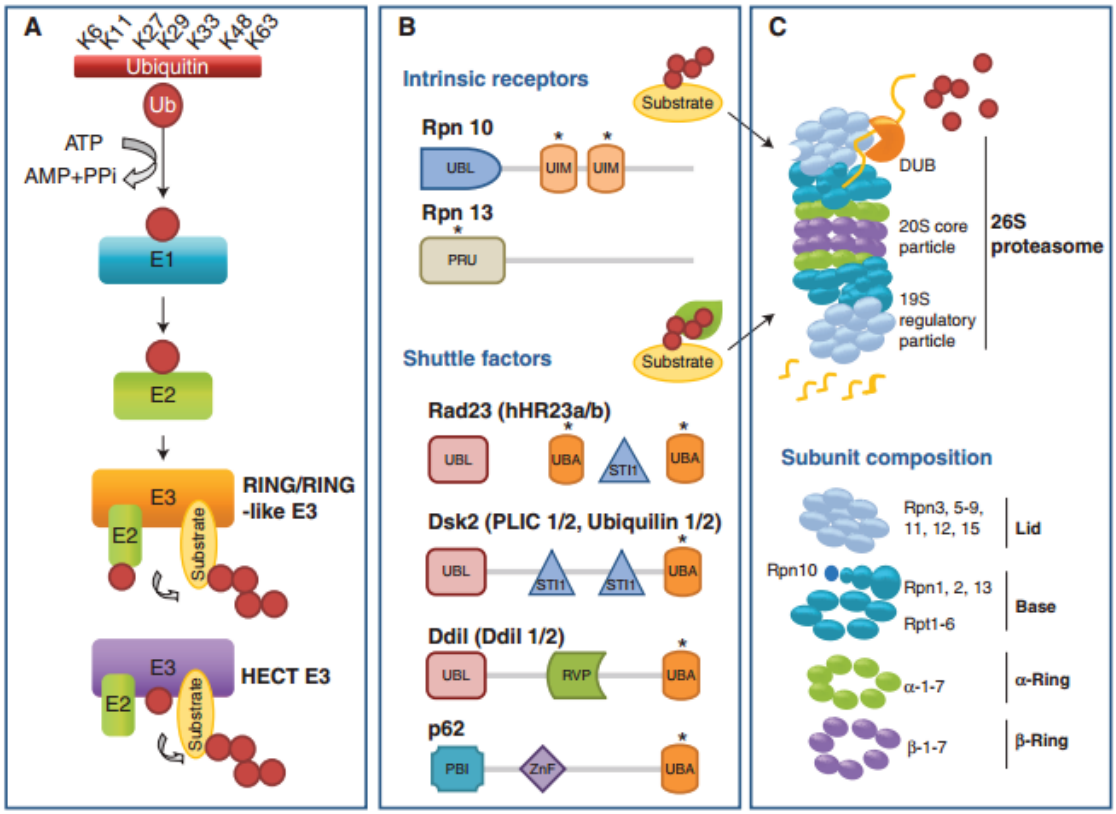

Figure 12: Components of the ubiquitin/proteasome system (UPS). **A)** Substrate ubiquitination occurs through a series of sequential reactions catalyzed by ubiquitin-activating (E1), conjugated (E2), and ligating (E3) enzymes. The transfer of ubiquitin by the two main families of E3 enzymes (RING/RING-like and HECT) is depicted. E3 RING enzymes catalyze the transfer of ubiquitin directly from E2 to the substrate, whereas HECT E3 enzymes (E3 HECT) accept activated ubiquitin in the form of a thioester from an E2 before transfering it to the substrate **B)** Proteasome intrinsic receptors and external shuttle factors allow the transfer of Ub-proteins to the 26S proteasome **C)** Structure and subunit composition of the 26S proteasome. See text for details. From Cold Spring Harb Perspect Biol. 2010 Dec;2(12):a006734.

The post-translational attachment of ubiquitins (Ub), repetitions of a single 76 aa polypeptide, directs eukaryotic proteins to a variety of fates and functions. Ubiquitination is best known for its role in the degradation of proteins, but it's also involved in internalization or lysosomal guidance, modulation of protein interactions, regulation of transcription, DNA repair, and propagation of transmembrane signaling [64].

Ub is covalently bound through a glycine carboxy-terminal to a lysine ε-amino group of a substrate. Proteins can be tagged by mono or poly-ubiquitination. Since Ub contains seven lysine residues, different chain topologies of polyubiquitination are described. The main signals that target substrates to proteasome clearance are tagged by K48 and K11 ubiquitination. Protein can be also ubiquitinated in multiple residues leading to multi-mono and multi-polyubiquitination [65].

Substrate ubiquitination occurs through the sequential action of 3 enzymes. The C-terminal carboxyl group of Ub is activated through an ATP-dependent process, which results in a thioester linkage with a cysteine of

E1 (ubiquitin activating enzyme). Activated Ub is then transferred to the active site of E2 (ubiquitin conjugatin enzyme). In the final step, E3 (ubiquitin ligase enzyme) catalyzes the transfer of a single molecule of ubiquitin to the substrate. (**Fig. 12A**) [66].

E3 is the most important enzyme in determining the specificity of substrate ubiquitination. More than 600 E3 enzymes are estimated to be encoded in the mammalian genome [64]. E3 enzymes can be classified according to the presence of a series of domains such as the "really interesting new gene" (RING or RING-like) or the **H**omologous to the **E**6-AP **C**arboxyl **T**erminus (HECT). The vast majority of E3s belong to the RING finger ubiquitin ligase, which comprises plant homeodomain (PHD), leukemia associated fingers proteins (LAP) and members of the U-box family. E3 enzymes with RING domain mediate a direct transfer of Ub from E2 to the substrate. Oppositely, in the case of HECT E3s, Ub is first transferred to a conserved Cys residue of the HECT domain and then attached to the substrate (**Fig. 12B**).

E3 enzyme-mediated cargo recognition occurs through two possible strategies: E3 enzyme can recognize a "degron" or degradative signal. However, in other cases, the specific recognition can be modulated by the chaperone system HSP70/HSP90, like for the member of the U-box family, CHIP.

Once tagged, ubiquitinated-substrate proteins are directed to the proteasome for degradation. Ub-proteins bind directly to the ubiquitin receptors of the 19S particle of the proteosome or alternatively, interact with shuttle factors. These factors can be stable subunits of the proteosome (i.e. RPN10 and RPN13) or transiently associated shuttle proteins (i.e. p62, RAD23, Dsk2, Ddi1). After the binding to a receptor, deubiquitinating enzymes (DUBs) like RPN11, disassemble the polyubiquitin chains before the substrate can have access to the proteasome proteolytic core (**Fig 12B**). The 26S proteasome is a large ATP-depending multimeric protease complex that consists of two subunits: the catalytic 20S core particle (CP) and the 19S regulatory particle (RP) (**Fig 12C**). The CP subunit resembles a barrel and it is organized in two outer α-rings and two inner β-rings. The α-rings serve as a gate for the substrate entry, while the β-rings form the proteolytic chamber. The entrance of the substrate into the inner surface of the CP subunit is regulated by the RP subunit, which controls the opening and closing of the α-rings gate. RP particle consists of 19 subunits organized in lid and base structures. The base, in turn, comprises 4 non-ATPase subunits and six AAA' ATPase subunits, which provide the energy for the substrate deubiquitination and unfolding, as well as the opening of the α-rings gate.

5.3 Autophagy: between homeostasis and stress. Overview of Autophagy

The term autophagy derives from the Greek auto "self" and phagein "to eat". It refers to any cellular degradative pathway which eventually leads to the delivery of cytoplasmic components to the lysosome [67].
On the basis of the delivery mechanism involved, autophagy can be classified into: Microautophagy, Chaperone-mediated autophagy (CMA) and Macroautophagy. The three forms of autophagy are summarized in **Fig. 13**.

Microautophagy implies a direct lysosomal uptake of cytoplasm by invagination. This process is ATP-dependent and is induced by lipids and lipid-interacting proteins. During microautophagy, the formation of a tubular shape invagination (termed autophagic tube), controlled by the GTPase Vps1p, has been reported. Vesicles, which are the equivalent to autophagosomes in macroautophagy, develop at the top of the tubes. Afterwards, they expand within the autophagic tube and finally fuse with the lysosomal membrane [68].

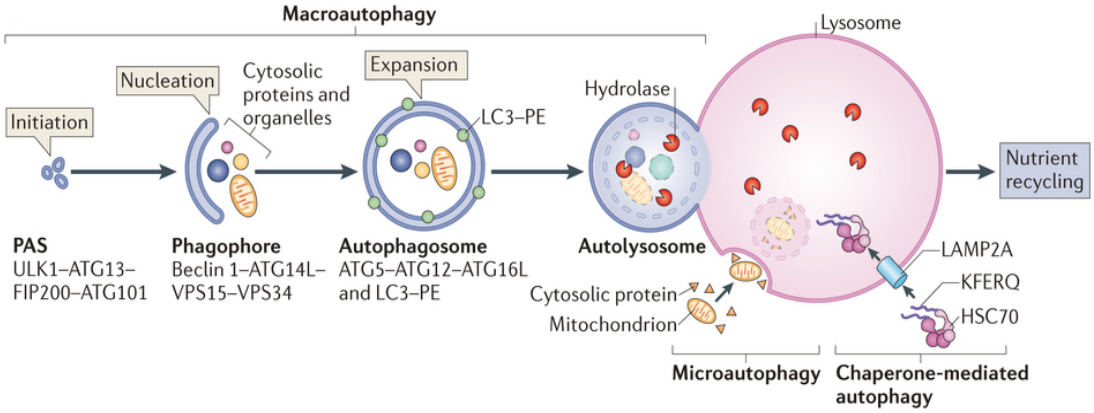

Figure 13: Overview of mammalian autophagy. Depiction of the three types of lysosomal degradative-autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). In macroautophagy, proteins, organelles and cytosolic materials are sequestered in double membrane vesicles (autophagosome) that finally fuse with the lysosome. In microautophagy, small portions of the cytoplasm are engulfed by lysosomal invagination. In CMA, the pentapeptide KFERQ-like present in certain proteins is recognized by the HSC70 chaperone. Recognition of this sequence directs proteins to lysosome for degradation. Adapted from Nat Rev Mol Cell Biol. 2015 Aug;16(8):461-72.

CMA is a catabolic pathway that targets a specific subset of cytosolic proteins to the lysosome for degradation. Proteins amenable to CMA need to possess a KFERQ or a KFERQ-like motif, which mediate the recognition by the chaperone heat shock cognate 70 (HSC70).
HSC70 along with its co-chaperons (HSP40, BAG1, HIP and HOP) brings the substrate protein to the lysosomal surface. The complex interacts with LAMP-2A, which acts as a receptor, transferring the chaperones and the target protein into the lysosomal lumen [69].

Macroautophagy (hereafter referred as autophagy) is a highly conserved process from yeast to humans. Regulated by the serial action of the autophagy proteins (ATGs), it consists in a course of events beginning with the selective or bulk sequestration of cytoplasmic components into double-membrane vesicles termed autophagosomes and followed by the fusion with lysosomes for the degradation of their cargo. While

ubiquitin-proteasome system (UPS) is specialized in the clearance of short-living proteins, basal autophagy is in charge of getting rid of dysfunctional/long-lived proteins and altered/damaged organelles. Likewise, a series of ATGs knock-out models evidenced the relevance of autophagy in the accumulation of cytotoxic depositions of aggregate-prone proteins. For this reason autophagy is being proposed as a basal quality control mechanism. In addition, autophagy acts in coordination with the UPS system for the clearance of ubiquitinated proteins [65, 70].

Differently from basal autophagy, induced autophagy is activated in response to a variety of stressors (e.g. Staurosporine, H_2O_2, ER-stress, chemo, radiotherapy and metabolic stress). Functionally, it seems in charge of dampening the stress-mediated cytotoxic effects. Alternatively, starvation-induced autophagy would be in charge of maintaining cell viability by providing the building blocks when external energetic sources are not available [71].

To date, autophagy has been involved in the regulation of many physiological processes, such as normal development, senescence, acclimatization, lifespan extension, immunity and defense against pathogens. On the other hand, its defects have been linked to myopathies, neurodegeneration diseases (such as Huntington's and Parkinson's disease), heart, liver, bowel pathologies and Cancer [72].

5.3.1 Autophagy in cancer

In 2000, Hanahan and Weinberg described a list of hallmark capabilities acquired by cancer cells to evolve from a normal cell to a neoplastic one. Cancer development depends on the progressive acquisition of defects in regulatory circuits controlling cell proliferation and cell death but also on the alterations in the homeostatic mechanisms [11].

The role of autophagy in tumorigenesis has been described with the metaphor of a double-edged sword because of its putative different effects according to the tumor stage. Broadly speaking, initiation and progression stages are buffered by autophagy while during progression, metastasis and therapy-resistance, autophagy seems to support cancer cell growth and survival. This image is being permanently questioned, being still impossible to answer whether autophagy favors or blocks cancer.

One of the first links between autophagy and tumor development was the observation that the master regulator protein Beclin1 (BECN1) was monoallelically deleted in many breast, ovarian and prostate cancers, thus suggesting autophagy was a tumor suppressor mechanism [73]. While *becn1* $^{-/-}$ mice die early in embryogenesis, *becn1*$^{+/-}$ mice show increased incidence of lymphomas, liver and lung carcinomas.[74] Subsequent studies documented mutations of *ATG2B*, *ATG9B*, *ATG*5, *ATG12*, *UVRAG* in gastric and colorectal cancers. In the same line, p62/Sequestosome-1, a multi-domain protein adapter participating in autophagy, is overexpressed in a large number of tumors. p62 is reported to promote tumor formation because it alters the NF-κB pathway. In an autophagy-deficient context, a raise in p62 fuels a positive loop involving ROS and DNA damage, which can also contribute to carcinogenesis [75].

On the other hand, mice with a systemic mosaic of *ATG5* deletion or with a liver-specific knock-out of *ATG7* induce only the appearance of liver benign adenomas, implying that the ablation of these two genes is only able to induce the very earliest stages of tumor development in some specific organs [76]. Furthermore, recent studies suggest that the loss of *BECN1* in human cancers is associated to the deletion of the neighboring *BRCA1* gene, a well-known tumorsuppressor, thus weakening the evidences of Beclin-1 as a tumor suppressor.[77]

Autophagy has been recently considered also a mechanism by means of which, cancer cells escape from immunosurveillance. The suppression of autophagy is reported to dampen the release of ATP, one of the finding-me signals for macrophage and dendrocytes (DC) precursors [6].

The role of autophagy in an established tumor is less controversial. The structure of a proliferative solid tumor comprises a proliferative periphery where oxygen and nutrients are largely accessible (but transiently unavailable due to the chaotic and heterogeneous tumor vascularization) and a central core, characterized by a recurring nutrient depletion and hypoxia. The core of the tumor is also characterized by the presence of quiescent cells. In such conditions, cancer cells employ autophagy as a mechanism to counteract the metabolic stress and sustain viability [78].

5.3.2 The core pathway of canonical mammalian autophagy

Autophagy relies on a sequence of ATP-dependent dynamic membrane modifications mediated by the autophagy related proteins (ATGs).

Genetic screening in yeast has identified more than 30 ATGs, but only 15 are actually part of the central core of autophagy. A schematic overview of the canonical mammalian autophagy is depicted in **Fig 14**. Autophagy has been classically broken down in different steps: initiation (or induction), nucleation, elongation/completion and maturation/degradation.

In mammalians, autophagy begins with the formation of the isolation membrane (also called phagofore). The source of the autophagosome is a debatable question. Several theories have been proposed, but two are predominant:

- The autophagosome is crafted *de novo* by the ATG9-dependent addition of lipids from mitochondria, plasma membrane and Endoplasmic Reticulum [79].
- A subdomain of ER is used for the formation of the phagophore that then expands exploiting closely interconnected ER domains [80].

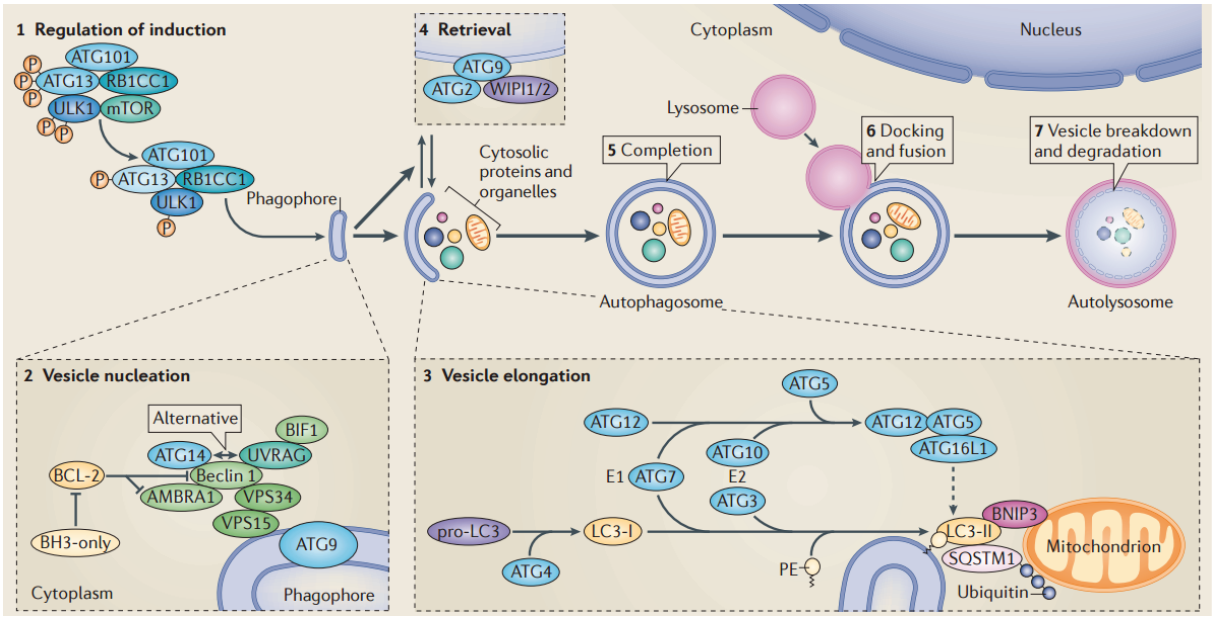

Figure 14: Overview of the mammalian autophagy core machinery. Autophagy is initiated by the formation of the phagophore or isolation membrane, an event regulated by the ULK-complex (1). Next step is the nucleation, which requires the assembly of a multimeric complex characterized by the presence of Beclin1/VPS34(PI3K3C) (2). Vesicle elongation is mediated by two ubiquitin-like conjugation systems, producing ATG5:ATG12 and lipidated LC3 (LC3-II), respectively (3). Phagophore expansion (retrieval) is mediated by WIPI 1/2 and ATG9 (4). When the mature autophagosome is completely formed (completion) (5), it fuses with the lysosomal compartments (docking and fusion) (6), ultimately leading to the formation of the auto(phago)lysosome. Finally, the internal membrane coming from the autophagosome together with the cargo will be degraded by lysosomal proteases (7). See text for details. Adapted from Nat Rev Mol Cell Biol. 2014 Jan;15(1):65-74.

Induction

The induction phase depends primarily on the ULK complex, a pretty stable complex regardless of nutrients abundance. The core of the ULK complex are, two serine/threonine kinases known as unc-51-line kinase 1 (ULK1) and ULK2 (two homologs of yeast ATG1). In addition, the complex contains ATG13, FIP200 and ATG101. The ULK complex functions integrating upstream signals coming mainly from the mTORC1 pathway. When mTORC1 is switched off by TSC1/2 heterodimers, ULK1 autophosphorylates itself and at specific residues on ATG13 and FIP200 and localizes to the phagophore. Atg101 binds and stabilizes Atg13, and is required for autophagy, at least in mammal.

While a few interactions between ULK and the downstream ATG proteins (e.g. ATG5-complex) have been identified, it's unresolved how the ULK complex and the beclin-1/PI3K3C module are interconnected.

Vesicle Nucleation

The nucleation and assembly of the rising phagofore is dependent on the Beclin1/hVPS34, a complex containing Beclin1 (ATG6 in yeast), hVPS34 (also called class III phosphatidylinositol 3-kinase (PI3K3C)), a regulatory protein kinase called p150 (also termed hVps15), Barkor (homolog of Atg14 in yeast). In addition, the Beclin-1/hVPS34 complex bound to UVRAG and Rubicon is also involved in the maturation and endosomal trafficking, (See below).

Phosphatidylinositol 3-phosphate (PtdIns3P) is the enzymatic product of hVPS34 and it's essential for the nucleation stage. The activity of PI3K3C is controlled by positive and negative regulators. A positive regulator is VMP1 (also called TMEM49), a transmembrane protein participating in the translocation of Beclin-1, AMBRA1, MYD88 and Bif1 to the nascent autophagosome. On the opposite, the ER fraction of anti-apoptotic BCL-2 family members is known to play a negative role over nucleation.

The nucleation step is physically mediated by the ATG18 analog in mammals WIPI-1/2, which belongs to the WIPI family of phospholipid binding effectors and interacts with PtdIns3P. WIPI2 functions controlling the transport of ATG9 from the phagophore to the endosome/Golgi localization. ATG9, which is the only identified transmembrane ATG, co-localizes with LC3 and Rab7 under starvation and it's involved in carrying lipids to the nascent autophagosome.

Elongation.

During the elongation phase, the Beclin-1 complex recruits ATG12-ATG5:ATG16L multimeric complex and LC3-I (analog of ATG8 in yeast) to the nascent autophagosome. Soon after their synthesis, ATG12 and ATG5 are covalently conjugated through an ubiquitin-like system. In the first place, ATG12 is activated by ATG7 (E1-like) and then conjugated by ATG10 (E2-like) to ATG5. ATG12-ATG5 interact with ATG16L and participate in the lipidation of LC3-I. LC3-I, which routinely resides in the cytoplasm, is cleaved by ATG4B and then, bound to the polar head of phosphatidylethanolamine (PE) in a reaction that requires ATG7 (E1-like), ATG3 (E2-like) and finally, the complex ATG12-ATG5:ATG16L (E3-like). The lipidated form (termed LC3-II) is important for expanding and lengthening the autophagosome but also for cargo recognition. The interactome of LC3-II includes p62/Sequestosome 1 (SQSTM1) through a region termed LC3-interacting region (LIR). P62 has been proposed to function as well as a cargo, regulating the packing and delivery of poliubiquitinated proteins and dysfunctional organelles [81]. LC3-II is associated with both the inner and outer nascent autophagosome membrane. When the double-membrane is formed, the ATG12-ATG5:ATG16L complex leaves the autophagosome, while only the LC3-II associated with the autophagosomal cytosolic surface is cleaved by ATG4B and recycled.

Maturation and degradation

The maturation/degradation phase is dependent on the interaction between autophagosome and lysosome, leading to the formation of a hybrid organelle, termed autophagolysosome. The autophagosome are transported in a dynein-dependent manner along microtubules to the endosome or directly to the lysosome. The process of fusion is mediated by a series of soluble NSF attachment protein receptors (SNAREs), in particular Vti1b. Lysosomal hydrolases become active in response to the progressive acidification of the organelle. Finally, a concomitant degradation of the inner autophagosome membrane and the sequestered cytoplasmic content is observed. These phenomena will provide cells with a series of biomolecules that will be delivered from the autophagolysosome through several permeases.

Rubicon and UVRAG are two Beclin-1 interacting proteins that play a role in maturation. While Beclin-1:hVPS34:UVRAG up-regulate the process, the binding of Rubicon to this complex plays a negative role [82]. In this sense, Rubicon overexpression or depletion is reported to cause a p62 accumulation or degradation, respectively [83]. In addition, overexpression of Rubicon seems to alter the acidification of LC3-II-associated vesicles.

5.3.3 Alternative/non-canonical Autophagy

Contrary to the canonical autophagy, the non-canonical autophagy does not require the same ordered activation of ATGs to trigger the degradation of cytosolic material into the lysosomes [84]. To date, different forms of non-canonical autophagy have been reported under specific conditions, for instance in response to pharmacological compounds or in the context of peculiar knock-out models.

At least three main alternative autophagic pathway can be distinguished: Beclin-1/hVPS34-independent autophagy, ATG5/ATG7-independent autophagy and ULK-independent autophagy.

Beclin-1/ hVPS34 independent autophagy. A non-canonical autophagy has been reported in the absence of the nucleation Beclin-1/hVPS34 activity in several cancer cell lines facing different apoptotic-induced stressors or autophagy-inducers. This form of alternative autophagosome formation still requires the ubiquitin like protein conjugation system and it's insensitive to 3-MA (the most used PI3K3C inhibitor). Staurosporine (STP), MK801 and etoposide have been found to induce Beclin-1 independent autophagy in cortical neurons [85]. The same phenomenon has been shown in HeLa cells treated with Z18, a compound that targets the BH3 binding groove of BCL-X_L/BCL-2, and in RAW 264.7 macrophage cells treated with H_2O_2. Likewise, Beclin-1 independent autophagy were identified in response to resveratrol in MCF-7 and to neurotoxin MPP+ in dopaminergic neuronal cells [86].

Interestingly, human cancer cell lines exposed to Gossypol or Arsenic Trioxide undergo an hVPS34-dependent and Beclin-1 independent form of autophagy. This finding was confirmed by some *in vivo* models of mature sensory neurons, lymphocytes, heart and liver, where the autophagosome formation can occur in the absence of hVPS34 and its PI3K3C activity.

ATG5/ATG7-independent autophagy. This form of autophagy has been observed in MEFs *Atg 5* $^{-/-}$ and *Atg7* $^{-/-}$ upon prolonged etoposide exposure. Under this treatment, ultrastructural analysis by transition electron microscopy revealed the presence of all the stages of autophagosome biogenesis: isolation membrane, autophagosome, amphisomes (pre-autolysosomal vacuoles) and autophagolysosome. Based on the unusual lamination of the membrane forming the phagofore, it would seem that the trans-Golgi compartment and endosomes are the preferential source for this type of autophagy [87].

This form of non-canonical autophagy is dependent on ULK-1/2 complex and on the nucleation activity of Beclin1/hVPS34 complex, but independent of the ubiquitin like protein conjugation systems (LC3, ATG5 and ATG7) and ATG9. The GTPase Rab9 is necessary for the autophagosome elongation. ATG5-independent autophagy seems specifically involved in mitophagy during erythrocyte maturation, suggesting that both canonical and non-canonical pathways may contribute to this form of organelle-specific degradation [88].

ULK-independent autophagy: A form of autophagy that bypasses the canonical ULK induction has been reported upon glucose starvation and ammonia treatment. Although *Ulk1/2* $^{-/-}$ double knock out cells are selectively impaired in the autophagy response to amino acid deprivation, autophagy is normally engaged during glucose starvation. Following glucose withdrawal, non-transformed cells employ alternative source of energy. In this context, amino acids are the preferred catabolic substrate, at least until AMPK-dependent β-oxidation is activated. Ammonia is the main byproduct of an increase catabolism of nitrogenous molecules. Both WT and *Ulk1/2* DKO, subjected to low dose of ammonia (2-5 mM) activates autophagy in a ULK1/2 and mTOR independent fashion [89]. In another study, an independent group has identified FIP200-binding domain (FBD) in ATG16L. FBD represents a link between the ULK and the ATG5 complexes. Interestingly FBD is necessary for the ULK-dependent autophagy upon amino acids deprivation but dispensable for

autophagy activation during glucose deprivation [90]. These reports also arise the possibility that autophagosome formation can occur in the absence of the AMPK-mTOR-ULK route of initiation.

5.3.4 Regulation of autophagy

5.3.4.1 mTOR: the gatekeeper of metabolism and autophagy signaling

Autophagy is part of the metabolic intertwining circuits responsible of maintaining a balance between anabolism and catabolism. According to this fact, autophagy is sophisticatedly controlled by metabolic and energetic sensors such mTOR and AMPK.

The mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K) family. mTOR was first described as the target of rapamycin, a macrolide produced by *Streptomyces hygroscopicus* that gained attention for its anti-proliferative properties.

mTOR is part of two complexes: mTOR complex 1 (formed by mTOR, RAPTOR, mLST8, PRAs40) and complex 2 (formed by mTOR, RICTOR, mlst8 and sin1). They are clearly differenced by their components and functions. mTOR complexes display different sensitivities to the inhibitory actions of rapamycin. Furthermore, they are regulated by differential inputs and initiate distinct downstream signaling pathways. The mechanism of action of rapamycin it's not fully understood. However, it has become clear that rapamycin forms a gain-of-function complex with FK506-binding protein (FKBP12) protein, blocking mTORC1 (but not mTORC2 complex) through a direct interaction with this complex.

While mTORC2 is involved in the regulation of several kinases (e.g AKT and PKC) and can still indirectly regulate autophagy, mTORC1 acts as a rheostat, sensing the energetic status of the cell, modulating the proliferation and cell growth. It represents the converging hub of several pathways, integrating important intracellular and extracellular information from growth factors, stress, energy status, oxygen, and amino acids level. In this sense, it is responsible of controlling anabolic (including protein and lipid synthesis) and catabolic (including autophagy) processes [91].

Most of the upstream regulators of mTORC1 converge on the heterodimers of tuberous sclerosis 1 (TSC1) and TSC2 proteins, which negatively regulate mTORC1. TSC1 and TSC2 function like a GTPase activating protein (GAP) on the Ras homolog enriched in brain (RHEB) GTPase. The GTP-bound RHEB, which normally interacts and stimulates mTORC1 kinase activity, is converted into its inactive GDP-bound state by the TSC1/TSC2 complex. The upstream signals transduced by TSC1/2 include growth factors (insulin, IGF1, etc.) and several stresses among which, hypoxia, nutrients withdrawal, and DNA damage.

Under low energy and hypoxia, mTORC1 is repressed by adenosine monophosphate-activated protein kinase (AMPK) and REDD1, respectively. AMPK represents an important sensor of stress and the overall energy decrease. This protein senses changes in the AMP/ATP ratio. This situation is commonly found in response to glucose deprivation. In addition, AMPK can induce autophagy by direct phosphorylation of ULK1, as well as by the inhibition of mTORC1 through phosphorylation of TSC2 and RAPTOR. On the other hand, REDD1 is transcriptionally induced by HIFα during hypoxia, activating TSC2 in a still poorly understood manner.

mTOR regulates autophagy with a direct phosphorylation of ATG13 and ULK1/2. Interestingly after a prolonged period of starvation, mTOR is reactivated due to the release of nutrients from the autophagolysosome, a feedback that avoids excessive and prolonged autophagy. Both S6K1 (one of the most important downstream targets of mTOR along with E4-BP1) and the death associated protein 1 (DAP1) participate in this regulation, but the exact target of DAP1 still needs to be addressed [92]. The regulatory mechanism of mTORC1 on autophagy are depicted in **Fig. 15**.

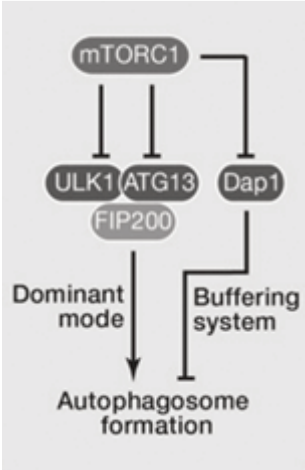

Figure 15: Regulation of autophagy by mTORC1. mTORC1 directly inhibits autophagy by the phosphorylation of specific nucleation complex proteins, such as ULK1 and ATG13. Dap1 is part of the negative feedback that reactivates autophagy after prolonged starvation. See text for details. Adapted from Cell. 2012 Apr 13;149(2):274-93.

In addition, mTORC1 is strictly tuned by the level of amino acids, particularly leucine and arginine. Amino acids act independently from TSC1/2 and require the RAGs GTPase and the assembly of the so called Ragulator on the lysosomal surface, where mTORC1 translocates in order to interact with RHEB.

5.3.4.2 P53: the Janus of autophagy regulation

The p53 regulatory network on autophagy is multifaced: physiological levels of p53 dampen basal autophagy while stress-induced activation of p53 upregulates autophagy.

In the absence of stress, cytoplasmic p53 displays anti-autophagic properties. This effect is exploited by p53 deficient cancer cells to maintain ATP levels in starvation. Accordingly, human *p53* $^{-/-}$ colon cancer cells are characterized by presenting greater levels of autophagy that can be reduced by re-introduction of wild-type (WT) p53. The mechanism behind this effect is not clear, but it seems independent of p53 transactivatory activity [93].

Upon DNA damage, stabilized p53 translocates to the nucleus where induces the transcription of pro-apoptotic genes but also of positive regulators of autophagy: AMPK β1/β2, damage-regulated autophagy modulator (DRAM), PTEN, TSC2, sestrins1/2.

5.3.4.3 Regulation of autophagy by Bcl-2 family proteins

Anti-apoptotic BCL-2 family members function as negative regulators of autophagy. For instance, this is the case of Beclin-1, which possesses a BH3 domain with lower affinity for the anti-apoptotic BCL-2 members than the other BH3-only proteins. Through these interactions autophagy and apoptosis are regulated. At the ER level, the interaction of Beclin-1 with BCL-2, BCL-X_L or MCL-1 is preventing the assembly of Beclin-1/VPS34 complex, a step necessary for canonical autophagy. In cells subjected to starvation, the BCL-2/Beclin-1 complex is stabilized by the nutrient-deprivation autophagy factor-1 (NAF-1). Knockdown of NAF-1 diminishes the binding of BCL-2 to Beclin-1 and elicits autophagy [94]. Alternatively, Beclin-1:BCL-2 interaction can be disrupted by the JNK-mediated phosphorylation of Bcl-2 [95]. On the contrary, some BH3-only proteins (BNIP3L, BAD, NOXA, PUMA and BIK) compete with Beclin-1 for the binding with the BCL-2 family members. The release of Beclin-1 from this complex initiates autophagy [96].

6 Crosstalk between autophagy and other cell death modalities

6.1 Interlinks between autophagy and apoptosis

The crosstalk between autophagy and apoptosis has recently become an extensive subject of research. The way autophagy and apoptosis are interconnected is context and cell line-dependent. Autophagy can be engaged in parallel with apoptosis as an attempt to dampen cell death. Alternatively, autophagy can be a legitimate means of self-killing (autophagic cell death) or engage an alternative modality of cell death, for instance, apoptosis.

Three are the main possible scenarios:

1. Autophagy and apoptosis occur as two independent processes.
2. Autophagy regulates/primes apoptosis.
3. Apoptosis regulates autophagy.

6.1.1 Autophagy and apoptosis occur as two independent processes

In this setting autophagy is engaged independently of apoptosis and can antagonize the deleterious effects of apoptosis or, oppositely, synergize with apoptosis in inducing lethality.

Many apoptotic triggers and stresses are reported to activate both autophagy and apoptosis. In some cases, autophagy is activated as a pro-survival program that attenuates the toxic effects of the pro-death pathways. For example, autophagy is reported to maintain ER function during ER stress, through the clearance of aggregates and misfolded proteins, to preserve genomic integrity during metabolic stress or DNA damage and to buffer energy depletion facing serum or glucose starvation [97].

In other settings, autophagy and apoptosis cooperate to ensure the dismantling of the cell. In this regard, if one of the two machineries is blocked, the other may gain control of the process. This could also explain why several validated forms of autophagic cell death were reported in cells where the apoptotic machinery has been disabled. However, there are multiple models where the contribution of each process in the final fate of the cell is well defined. For instance in T-lymphocytic leukemia cells, autophagy and apoptosis activated in response to arsenic trioxide are both contributing to the final death outcome. Similar evidences are reported in kaposi's sarcoma treated with imatinib, PC3 prostate cancer cells treated with MG132 (a proteasome inhibitor) and HL-60 cells subjected to vitamin K2 [97]. *In vivo*, autophagy is involved in the post-lactation regression of mammary gland in mice, a process perturbed by ablation of *Atg7*, but relying on the apoptotic machinery [98].

A similar scenario is illustrated by DRAM (damaged-regulated autophagy modulator), a stress-inducible p53-dependent gene. In response to stress, p53-induced DRAM triggers autophagy and is essential for p53-driven cell death. The mechanism to elicit autophagy is unknown, only its lysosomal trans-membrane localization suggests a pro-autophagic function. Curiously, in the absence of p53, DRAM is merely promoting autophagy without affecting cell viability.

Under certain circumstances autophagy enables several apoptotic features. In this instance, autophagy assists the apoptotic program without actively participating in cell dismantling. For example, autophagy-dependent maintenance of ATP levels may not alter the cell death fate but be necessary for the appearance of certain

apoptotic features, such as phosphatidylserine exposure, membrane bebbling, and apoptotic body formation/engulfment. [97]

When apoptosis is disabled, autophagy may gain control. A good example is represented by the cell lines generated from the bone marrow of *Bax* $^{-/-}$/*Bak* $^{-/-}$ (DKO) mice, which fail to undergo apoptosis when subjected to IL-3 withdrawal (the default response of the WT counterpart). In this condition, DKO cells enter instead in a long autophagic process that causes a prominent reduction of size. The inhibition of autophagy in this experimental paradigm rescues such phenotype [99].

6.1.2 Autophagy primes/regulates apoptosis

In some cases, autophagy can prime caspase-dependent cell death and, as a result, the blockage of autophagy prevents or delays apoptosis. This is the case of light-damaged mouse retinas or photoreceptor cells subjected to H_2O_2, where autophagy shows to be necessary for apoptosis induction [100]. Similarly, cerebellar granule cells subjected to low K^+ are protected from caspase activation and cell death by the pharmacological inhibition of autophagy [101].

Autophagy-dependent apoptosis does not always require the completion of the autophagic program since in multiple paradigms the inhibition of the autophagosome formation triggers cell survival. In this sense, the autophagosomes could act as a site of assembly. For instance, the autophagosome is a reported docking site for the assembly of caspase-8-dependent DISC. In this setting, caspase-8 interacts with FADD and ATG5, but also to colocalize with LC3 and p62. Another similar example comes from fibroblastic cell lines undergoing cell death by SKI-I, a pan-sphingosine kinase inhibitor, or bortezomib, a proteasome inhibitor. In these settings, the inhibition of the early stages of autophagy (in *Atg3* $^{-/-}$ and *Atg5* $^{-/-}$ MEFs) reduces the activation of caspase-8 and -3, whereas the inhibition of the last steps of autophagy (by means of bafilomycin A1) triggers greater levels of apoptotic cell death [102]. In other instances, the ongoing autophagy can exacerbate the apoptotic cell death through the consumption of caspase inhibitors (IAPs) or of pro-survival factors. Apoptotic cell death of fly oocytes is a direct consequence of the autophagy-dependent degradation of IAPs, dBruce [103].

On the other hand, ATG proteins could also trigger a lethal apoptotic signal despite of their autophagic functions. Interesting regulatory interlinks are reported for ATG12 and the ATG12-ATG3 conjugated complex. ATG12 has been recently implicated in the induction of intrinsic apoptosis through the binding of several anti-apoptotic BH1-BH4 proteins, such as MCL-1. This interaction is governed by a BH3 domain-like sequence motif within a disordered region preceding the ubiquitin-like domain of ATG12 [104]. ATG12 is usually conjugated with ATG5 to form ATG12-ATG5, which is the E3-like element responsible of the LC3 lipidation. In 2010, a new function of ATG3 as a conjugation target of ATG12 was reported. The final conjugation product ATG12:ATG3 had no visible effects on autophagy since its disruption did not cause any change in the levels of starvation- or rapamycin-driven autophagy. Nonetheless disruption of ATG12-ATG3 complex triggered an expansion of the mitochondrial mass and protected cells from cell death mediated by mitochondrial pathways, including intrinsic apoptosis [105].

6.1.3 Apoptosis regulates autophagy

The first evidence of apoptosis regulating autophagy came after the discovery of the interaction of several anti-apoptotic BCL-2 family members with Beclin-1. As described before, the anti-apoptotic BCL-2 family members (e.g. BCL-2, BCL-XL, MCL-1) downregulate autophagy through the binding to Beclin-1, thus preventing its association with VPS34. Contrarily, the BH3-only proteins induce autophagy by disrupting such interaction. This is the case of BNIP3, a BH3-only with a clear function as a trigger of mitophagy [106].

Executioner caspases and other proteases (e.g. Calpains) have been reported to cleave and inactivate ATG proteins with the peculiarity of providing new pro-apoptotic properties to the cleaved forms. For instance, the caspase-dependent cut of Beclin-1 brings to the formation of a C-terminus fragment, which translocates to the mitochondria where it sensitizes cells to apoptosis [107]. In the same line, ATG5 cleavage by calpains produces a 24 kDa truncated product, which functions as a BH3-only protein and promotes apoptosis by antagonizing the pro-survival role of BCL-X_L [108]. On the contrary, caspase-3-mediated cleavage of ATG4D, one of the variant of ATG4, promotes rather than inhibits autophagy.

6.2 Interlinks between autophagy and regulated necrosis

The relationship between autophagy and necroptosis is context specific. Autophagy has been shown to promote or suppress necroptosis. In some settings, autophagy is required for RIPK1 degradation and the inhibition of necroptosis. Nonetheless, for example, cells subjected to obatoclax (also known as GX15-070, a potent inhibitors of anti-apoptotic BCL-2 family members) require autophagy for the engagement of necroptosis [98]. In the context of Parthanatos, DNA-damage PARP-1 hyperactivation leads to ATP depletion with activation of AMPK and subsequent promotion of autophagy. DNA damage-induced autophagy acts as a protective mechanism against necrotic cell death resulting from PARP-1 activation [109].

V OBJECTIVES

The main aim of this study was to comprehend the implication of autophagy in cell death modulation. Two different and divergent experimental approaches were employed: starvation media (SM) and PES.

SM represents a way to mimic metabolic stress. The adaptation to nutrients and growth factor deprivation represents an acquired phenotype relevant for cancer progression and metastasis. The objectives of the first part address the effect of SM on cell death. The specific objectives were:

1) To evaluate cell death and engagement of autophagy in response to severe starvation by SM of multiple tumor and non-tumor cell lines
2) To elucidate the mechanism of cell death in cells subjected to SM, focusing on the involvement of apoptosis and necrosis.
3) To assess the contribution of canonical autophagy on SM-driven apoptotic cell death.
4) To assess the contribution of canonical autophagy on apoptosis triggered by SM in combination with pro-apoptotic compounds.

PES is a compound well known to block autophagic flux and other proteohomeostatic systems. The objectives of this part of my study were:

1) To study the effect of PES on cell viability in tumor and non-tumor cell lines.
2) To investigate the mechanism of cell death induced by PES and thus, the involvement of the three main cell death subroutines (apoptosis, autophagy and necrosis).
3) To elucidate the role of p53 in PES-driven regulated necrosis.
4) To elucidate the involvement of ROS in PES-driven regulated cell death
5) To study the complex p53-ROS partnership in the induction of PES regulated necrosis.
6) To shed light on the paradoxical protective function of BAX in PES-induced regulated necrosis.

VI PAPERS

1 Autophagy exacerbates caspase-dependent apoptotic cell death after short times of starvation

G Model
BCP 12390 No. of Pages 14

ARTICLE IN PRESS

Biochemical Pharmacology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

Autophagy exacerbates caspase-dependent apoptotic cell death after short times of starvation

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ARTICLE INFO

Article history:
Received 30 June 2015
Accepted 29 September 2015
Available online xxx

Chemical compounds studied in this article:
3-Methyladenine (Pubmed CID 1673)
Camptothecin (Pubmed CID 24360)
Etoposide (Pubmed CID 36462)
7-Bromoindirubin-3-oxime (Pubmed CID 11846148)
Necrostatin-1 (Pubmed CID 282833)
Q-VD-OPh (Pubmed CID 11237609)
2-Phenylethynesulfonamide (Pubmed CID 327653)
Staurosporine (Pubmed CID 44259)

Keywords:
Apoptosis
Autophagy
Short-term starvation
Mitochondrial permeabilization
Caspases

ABSTRACT

Autophagy is generally regarded as a mechanism to promote cell survival. However, autophagy can occasionally be the mechanism responsible of cell demise. We have found that a concomitant depletion of glucose, nutrients and growth factors provoked cell death in a variety of cell lines. This death process was contingent upon caspase activation and was mediated by BAX/BAK proteins, thus indicating its apoptotic nature and the engagement of an intrinsic pathway. In order to abrogate autophagy, 3-methyladenine (3-MA), BECLIN-1 siRNA and *Atg5* knock-out (Tet-Off type) approaches were alternatively employed. Irrespective of the procedure, at short times of starvation, we found that the ongoing autophagy was sensitizing cells to the permeabilization of the mitochondrial outer membrane (MOMP), caspase activation and, therefore, apoptosis. On the contrary, at longer times of starvation, autophagy displayed its characteristic pro-survival effect on cells. As far as we know, we provide the first experimental paradigm where time is the only variable determining the final outcome of autophagy. In other words, we have circumscribed in time the shift transforming autophagy from a cell death to a protection mechanism. Moreover, at short times, starvation-driven autophagy exacerbated the apoptotic cell death caused by several antitumor agents. In agreement with this fact, their apoptotic effects were greatly diminished by autophagy inhibition. The implications of these facts in tumor biology will be discussed.

1. Introduction

Insufficiently vascularized solid tumors commonly suffer from transient periods of nutrient, growth factors and oxygen restriction. An analogous situation is found in myocardial and brain tissues after ischemia. These severe forms of starvation impart a strong metabolic stress, which is partially alleviated through the induction of macroautophagy [1]. Macroautophagy (hereafter referred as autophagy) is an exquisitely orchestrated catabolic process whereby portions of the cytoplasm are sequestered into double-membrane vesicles called autophagosomes. The fusion of these vesicles with lysosomes, that supply their hydrolytic machinery, will result in the formation of the autophagolysosomes. These degradative organelles will fuel the cellular metabolism through the recycling of biomolecules and, indirectly, contributing to the bioenergetic management. Severe forms of starvation

Abbreviations: 3-MA, 3-methyladenine; 7BIO, 7-bromoindirubin-3-oxime; PES, 2-phenylethynesulfonamide; AVOs, acidic vesicular organelles; AO, acridine orange; AIF, apoptosis-inducing factor; ATG, autophagy-related; baf A_1, bafylomicin A_1; CPT, camptothecin; cyt C, cytochrome C; COX IV, cytochrome oxidase IV; DEVD, Ac-Asp-Glu-Val-Asp-AFC; DKO, double knockout; Eto, etoposide; LC3, microtubule-associated protein 1 light chain 3; MEFs, mouse embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilization; NB, naphthol blue; PES, 2-phenylethynesulfonamide; p62, p62/SQSTM1; PtdInsKC3, phosphatidylinositol 3-kinase class III; PI, propidium iodide; RIP1, receptor-interacting protein 1; siRNA, small interference RNA; SM, starvation medium; STP, staurosporine.

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http://dx.doi.org/10.1016/j.bcp.2015.09.021
0006-2952/

employ autophagy as a restorative system whereby cells enter in a "self-canibalism" process aimed to supply the cell with the metabolic intermediaries needed to survive. Consistently, starved cells undergo a reduction of size, which can be partially attributed to the autophagic process [2].

Canonical autophagy is driven by a series of sequential steps controlled by a group of "autophagy-related" proteins (ATGs), which are highly conserved from yeast to humans [3]. Classically, the process is divided in four steps: initiation, nucleation, elongation and closure. The formation of autophagic vesicles, or nucleation, is regulated by the phosphatidylinositol 3-kinase class III (PtdInsKC3; Vsp34 in yeast), which is the target of 3-methyladenine (3-MA), one of the most employed pharmacological inhibitors of autophagy. This kinase forms a complex with BECLIN-1, the mammalian ortholog of the yeast ATG6, a reported haploinsufficient tumor suppressor [4]. The elongation of the autophagosomes relies on the proper activity of two ubiquitin-like protein conjugation systems: the protein light chain 3 (LC3, also known as ATG8) and ATG12. These systems are interdependent since the complex ATG12-ATG5-ATG16 is in charge of transferring molecules of phosphatidyl-ethanolamine to LC3-I, a cleaved form of LC3. In this way, the lipidated form of LC3-I (LC3-II) will be properly located at the growing autophagosomes [5]. Alternatively, autophagosomes can be assembled independently of proteins such ATG5 or BECLIN-1, thus indicating the existence of non-canonical forms of autophagy [6,7]. LC3-II has been proposed to function as a receptor for a selective substrate, p62/SQSTM1, which is degraded within the autophagolysosomes. In addition, the p62/SQSTM1 ubiquitin-binding domain is proposed to act as a receptor for ubiquitinated proteins and inclusion bodies, which will be directed to the autophagosomes [8]. Both LC3-II and p62/SQSTM1 are powerful markers to study the dynamics of the autophagic process, also known as "autophagic flux" [9].

The role of autophagy in regards to the final fate of a cell is a subject of intense debate. In 1972, type II or autophagy was considered one of the three types of cell death, defined essentially on morphological grounds. For a while, the mere observation of a dying cell with a vacuolated cytoplasm was sufficient to conclude that this cell was dying by autophagy. Nonetheless, growing amount of data evidenced that autophagy cooperated or enabled alternative subroutines of cell death [10]. Therefore, the detection of autophagic features is not sufficient to discard the involvement of other pathways of cell death. Recent findings have established that autophagy, as a subroutine of cell death, is constrained to a few context- and cell-specific circumstances. Under these precise situations, the Nomenclature Committee of Cell death (NCCD) is favoring the use of the term "autophagic cell death" instead of the misleading, autophagy. Finally, it is worth to mention that autophagy is generally accepted as a pro-survival program triggered in response to unfavorable cellular environments [11]. Altogether, it seems that autophagy can lead cells to a broad range of cell fates. The specific molecular situations that will determine the consequences of activating autophagy are under study. Apoptosis is one of the most frequent subroutines of death engaged by starvation in cultured cells [1,12,13]. Intrinsic or mitochondrial apoptosis is marked by the mitochondrial outer membrane permeabilization (MOMP), which results in the release of cytochrome c (cyt C), the apoptosis-inducing factor (AIF) and other proteins from the inter-membrane space. Unleashed cyt C triggers the assembly of the apoptosome and the subsequent activation of the initiator caspase-9 upstream of the executioner caspase-3/7 [14]. On the other hand, AIF can promote forms of caspase-independent cell death [15]. Mitochondrial or intrinsic type apoptosis relies on BAX and BAK proteins and, therefore, the abrogation of these proteins is a good strategy to interrogate the involvement of this type of apoptosis in the cell death [16,17]. Last but not least, necrotic cell death has also been observed in specific cell lines or apoptotic-deficient ones undergoing starvation-driven autophagy [18,19]. Mounting evidences support the existence of diverse regulated forms of necrosis [20], one of which requires the kinase activity of RIP1 (receptor-interacting protein 1) and RIP3 [20,21]. Necrostatin-1, an inhibitor of the kinase RIP1, is a broadly employed tool to ascertain the participation of necroptosis in a specific lethal context [22].

We sought for a near-physiological manner to elicit autophagy such as the restriction of glucose, nutrients and growth factors. This model resembles the "*in vivo*" context of rapidly growing and insufficiently irrigated tumor. Under these restrictive settings, the role of autophagy on the final fate of cultures was interrogated. We found that a series of tumor and non-tumor cell lines, permanently deprived of glucose, nutrient and growth factor, underwent caspase-dependent mitochondrial-driven apoptosis. At short times, starvation-elicited autophagy was driving the mitochondrial permeabilization, the activation of caspases and cell death. On the other hand, at longer times, autophagy was mitigating cell lethality. Thus, these observations pull together the pro-survival and pro-death functions of autophagy under the same trigger, being the temporal frame the only determinant of the autophagic outcome. Moreover, we discovered that short-term starvation-driven autophagy sensitized cells to undergo apoptosis in response to several anticancer agents.

2. Materials and methods

2.1. Cells and cell cultures

Immortalized *Bax-/-Bak-/-* MEFs (DKO) and their wild type (WT) counterparts have their origin at late Prof. S.J. Korsmeyer's laboratory. *Atg5* Tet-Off MEFs m5-7 were gently supplied by Dr. Codogno's laboratory (originated at Prof. Mizushima's laboratory). PC3 and DU145 were kindly provided by Dr. Lupold's laboratory (James Buchanan Brady Urological Institute, Johns Hopkins University School of Medicine, Baltimore, Maryland, USA). SH-SY5Y, HeLa, MCF7 cell lines were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). SH-SY5Y, MCF7, HeLa and MEFs were maintained in DMEM and PC3 and DU145 in RPMI. All media were purchased from Lonza (Rockland, ME, USA) and contained a 5% volume of FCS (Biochrom AG, Berlin, Germany). 5 μg/ml Plasmocin™ (InvivoGen, San Diego, CA, USA) was used as the media antibiotic. General culturing conditions were 37 °C and a water-saturated, 5% CO_2 atmosphere. Culture dishes and other plastic disposable tools were supplied by VWR (Radnor, PA, USA) and Becton Dickinson (Franklin Lakes, NJ, USA).

2.2. Suppression of autophagy in Atg5 Tet-Off MEFs

To suppress autophagy, *Atg5* Tet-Off MEFs m5-7 [2] were maintained for 4 days in regular DMEM plus 40 ng/ml doxycycline hydrochloride (Sigma–Aldrich, St. Louis, MO, USA). Control populations of *Atg5* Tet-Off MEFs m5-7 undergoing autophagy were obtained by restoring the ATG5 expression in the former populations. Briefly, after subjecting the cultures to a regular growth medium plus Dox for 3 days, cultures were washed three times with phosphate-buffer saline (PBS) and cultured in Dox-free medium for an additional 24 h period.

2.3. Transfection of BECLIN-1 siRNA (siBec) or control siRNA (siC)

WT MEFs were subjected to two-rounds of reverse-transfection with the Lipofectamine® RNAiMAX Transfection Reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 3 μl of transfection reagent was used to reverse

Please cite this article in press as: P. Mattiolo, et al., Autophagy exacerbates caspase-dependent apoptotic cell death after short times of starvation, Biochem Pharmacol (2015), http://dx.doi.org/10.1016/j.bcp.2015.09.021

transfect 100 nM of siBecn1 (5′-CAGUUUGGCACAAUCAAUA-3′) or of non-targeting siC (5′-UAAGGCUAUGAAGAGAUAC-3′) from Sigma–Aldrich (St. Louis, MO, USA) diluted in Opti-MEM™ media (Life Technologies, Carlsbad, CA, USA) into 130,000 WT MEFs (22 mm diameter cell culture plate) growing in regular growing medium supplemented with 2.5% FBS in the absence of antibiotics. To perform a second round of transfection, the already transfected MEFs were harvested and subjected to another reverse-transfection following the previously described protocol. 24 h into the second round of transfection, FBS was increased to 5% in the presence of the transfection reagents and cells were allowed to recover for an additional 24 h period. Next, cells were plated to reach a 90% confluence at the time of starvation. To control the efficiency of siBecn1, whole cell extracts of WT MEFs transfected with siC or siBecn1 and subjected to starvation medium (SM), were obtained.

2.4. Cell starvation and treatment

To undergo the glucose, nutrient and growth factor starvation, WT, DKO and *Atg5* Tet-Off MEFs were plated in fresh media at 320 cells/mm^2 to reach a 90% confluence 24 h later. To be in the same conditions, HeLa cells were plated at 265 cells/mm^2. Owing to the great susceptibility of MEFs to detach from plastic, cell culture plates were pre-treated with 6 μg/ml poly-L-Lysine (Sigma–Aldrich, St. Louis, MO, USA) for 1 h. Glucose, nutrient and growth factor starvation was performed by rinsing cells once with PBS and incubating them in SM for the time specified in the figure legend. Composition of SM is 137 mM NaCl, 5.4 mM KCl, 0.25 mM NaH_2PO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 25 mM HEPES and 15.2 mg/l Phenol red. To pharmacologically inhibit autophagy, 3-MA (Calbiochem®, part of Merck KGaA, Darmstadt, Germany) diluted in SM at a final concentration of 10 mM, was applied to cell cultures for the times specified in the figure legends. To inhibit apoptosis, cell cultures were exposed to (3*S*)-5-(2,6-difluorophenoxy)-3-[[(2*S*)-3-methyl-1-*oxo*-2-[(2-quinolinylcarbonyl)amino]butyl]amino]-4-*oxo*-pentanoic acid hydrate (Q-VD-OPh) from Sigma–Aldrich (St. Louis, MO, USA) diluted in SM at the final concentration indicated in the figure legend. To inhibit necroptosis, cultures were exposed to necrostatin-1 (Tocris Bioscience, Ellisville, MI, USA) diluted in SM. WT and *Atg5* Tet-Off MEFs maintained either in regular full media or SM were further challenged with etoposide and staurosporine purchased from Calbiochem®, part of Merck KGaA (Darmstadt, Germany) or camptothecin and DMSO (as a control) from Sigma–Aldrich (St. Louis, MO, USA). To assay the effects of the necrotic inducers, 7-BIO and PES from Calbiochem® part of Merck KGaA (Darmstadt, Germany) were tested on WT MEFs. Unless otherwise stated, the non-listed reagents were from Sigma–Aldrich (St. Louis, MO, USA).

2.5. Western blot analysis

Cells were challenged with SM in the absence or presence of baf A_1 (Cayman Chemicals, Ann Arbor, MI, USA) for the times indicated in the figure legend. Whole cell extracts were obtained by lysing cells in a buffer containing 100 mM Tris/ClH pH 6.8, 1% SDS, 1 mM EDTA and the protease Inhibitor cocktail set III Calbiochem®, part of Merck KGaA (Darmstadt, Germany). Protein extracts were then boiled at 100 °C and sonicated for 20 s before being clarified by centrifugation. For cyt C subfractioning, cells treated as indicated in the figure legend, were lysed on ice for 5 min with a buffer containing 50 mM Tris pH 6.8, 150 mM NaCl, 1 mM EDTA, the protease Inhibitor cocktail set III and digitonin (Life Technologies, Carlsbad, CA, USA). Digitonin was adjusted to reach a final 0.04% (MEFs) or 0.016% (HeLa). Protein extracts were centrifuged at 4 °C for 5 min at 16,000 × *g*. Supernatant (cytosolic subfraction) was collected. After eliminating the traces of the cytosolic subfraction, pellet was lysed for 20 min on ice using a buffer containing 50 mM Tris pH 7.2, 150 mM NaCl, 0.5% NP40, 5 mM EDTA and the protease Inhibitor cocktail set III. Protein extracts were then clarified by centrifugation (pellet subfraction) and quantified by means of the DC Protein Assay (BioRad, Hercules, CA, USA). Volumes were calculated to equalize the protein load in SDS 12%-polyacrylamide gel electrophoresis. Following electrotransfer to 0.45 μm PVDF membranes (EMD Millipore part of Merck KGaA, Darmstadt, Germany), the following antibodies were applied: anti-caspase 3 (#9665) from Cell Signaling, anti-LC3B (L7543) and anti-p62 (P0068) from Sigma-Aldrich (St. Louis, MO, USA), anti-BECLIN-1 (NB110-87318) from Novus Biologicals (Littleton, CO,USA), anti-cyt C (sc-13156) from Santa Cruz Biotech (Dallas, TX, USA), and anti-COX IV (A21348) from Molecular Probes, part of Life Technologies (Carlsbad, CA, USA). Immunoblots were finally developed with the Immobilon™ reagent (Millipore, part of Merck KGaA, Darmstadt, Germany). Chemiluminiscence was recorded and densitometric analysis was performed by means of a Chemidoc™ apparatus and the Image Lab version 4.0.1 software from Bio-Rad (Hercules, CA, USA). Loading of each sample was assessed by staining the membranes during 5 min in a solution containing 10% methanol, 2% acetic acid and 0.1% of Naphthol blue black (Sigma–Aldrich, St. Louis, MO, USA). Then, membranes were de-stained in a 10% methanol and 2% acetic acid solution during 10 min. Membranes were allowed to dry and scanned. Western-blot quantification was the result of referring the densitometric signal of a given sample to the control stated in the figure legend and thus, obtaining the relative content. Graphics represent the average and standard error of the mean (S.E.M) of at least three independent western-blots.

2.6. Cell death and apoptosis assays

To determine cell death, cells were collected by trypsinization and centrifuged 3 min at 1500 rpm. Pelleted cells were resuspended in 5 μg/ml propidium iodide (PI) from Sigma–Aldrich (St. Louis, MO, USA) diluted in PBS. Following 15 min incubation at room temperature, cells were subjected to flow cytometry analysis. For subG1 analysis, treated cells were washed in PBS, fixed in 70% cold ethanol and incubated for a minimum of 24 h at −80 °C. Before staining, fixed cells were rinsed in PBS and resuspended in a PBS solution of 5 μg/ml PI and 100 μg/ml RNAse A (Boehringer Ingelheim, Ingelheim am Reihm, Germany). After 15 min incubation at room temperature, subG1 fractions were assessed by flow cytometry.

2.7. Caspase activation by DEVD-directed caspase activity

Caspase activity (DEVDase activity) was obtained by quantifying the fluorescence released from Ac-DEVD-afc (Enzo Life Sciences, Farmingdale, NY, USA) substrate after incubation at 37 °C in the lysed cultures. This method has been validated and described in our previous work [23]. Values after the subtraction of the basal DEVDase activity from an untreated control were plotted.

3. Results

3.1. Cells under glucose, nutrient and growth factor starvation show autophagic markers and caspase-dependent cell death

Cell death and autophagy are two common features of severely starved cultures. For this reason, we were interested in unraveling the relationship between cell death and autophagy under these conditions. A time course of cell death was elaborated using a series of ontogenically divergent tumor-derived cell lines (the

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breast cancer MCF7, the neuroblastoma SH-SY5Y, the cervix cancer HeLa and the prostate adenocarcinoma PC3 and DU145) subjected to Hank's balanced salt solution without glucose and fetal calf serum, hereafter referred to as starvation medium (SM). Of note, this buffer was partially mimicking "*in vitro*" the conditions of a tumor under an insufficient or null vascularization. Regardless of the time-dependent disparities, all the cell lines exposed to SM engaged into cell death (Fig. 1A). We focused our attention on HeLa cells because greater levels of cytotoxicity were evident at shorter times of SM treatment when compared to the other cell lines. To interrogate levels of autophagy in HeLa cells, we turned to commonly used markers of the autophagic flux, which are able to discriminate between blockage and induction of autophagy [24]. In this case, we explored the diminution of p62 and the levels of lipidated LC3 (LC3-II) in the presence or absence of the autophagic flux inhibitor bafilomycin A_1 (baf A_1). Baf A_1, an inhibitor of the vacuolar H^+ ATPase, was preferred over other lysosomotropic agents because of its lower cytotoxicity and nanomolar IC_{50}. The LC3-II increase in the presence of baf A_1 and the p62 reduction without baf A_1 evidenced that, from 6 h up to 16 h of starvation, HeLa cells presented greater levels of autophagy than in basal conditions (Fig. 1B). To corroborate our data through a third independent method, the acidic vesicular organelles (AVOs) were evidenced by acridine orange (AO). As expected, SM gradually increased the incidence of AO-stained orange-red AVOs in cultures of SM-treated HeLa cells while triggering a significant reduction of cell size (data not shown). Overall, these facts were strongly indicating the presence of active autophagy in HeLa cells. Cell death evaluation through propidium iodide (PI) staining revealed the presence of discrete amounts of cell death after 12 h of SM treatment, which sensibly increased to 35.6 ± 1.4% at 24 h and reached a maximum of 87.7 ± 3% at 48 h. Starvation can lead to both apoptotic and necrotic subroutines of cell death. To distinguish between these two possibilities, we assessed the protective effects of Q-VD-OPh, a broad inhibitor of caspases and necrostatin-1, an inhibitor of necroptotic cell death, on SM-driven cell death. While necrostatin-1 did not exert any overt protective effect (data not shown), the inhibition of caspases by Q-VD-OPh resulted in a significant protection of HeLa cells exposed to SM (Fig. 1C). Altogether, these results were suggesting that cells exposed to SM were undergoing an apoptotic cell death. According to the exposed role of caspases mediating starvation-driven apoptotic cell death, the activation of executioner caspases (caspase-3/7) in response to SM was interrogated throughout a 24 h period by measuring the DEVDase activity. HeLa cells responded to SM by inducing a significant activation of the executioner caspases already evident at 8 h of treatment (Fig. 1D). Therefore the coexistence of the apoptotic and autophagic programs were again confirmed. In parallel, we evidenced the cleavage of caspase-3 by western-blot, corroborating the results of the enzymatic activities (arrow, Fig. 1E). Finally, we detected that SM was triggering the appearance of subG1 DNA fragmentation in HeLa cells and that Q-VD-OPh was able to suppress it (Fig. 1F).

3.2. Glucose, nutrient and growth factor starvation leads to BAX/BAK-mediated apoptosis

Bax/Bak-/- double knockout (DKO) mouse embryonic fibroblasts (MEFs) are characterized by a disabled mitochondrial apoptotic pathway and, hence, they are protected facing most apoptotic stimuli, including serum deprivation, loss of attachment and growth factor withdrawal [16]. DKO MEFs are a valuable and broadly used tool to determine the involvement of mitochondrial or intrinsic apoptotic cell death in an experimental paradigm. DKO MEFs and their WT counterparts were subjected to SM and autophagy was assessed in a time-dependent fashion. In WT MEFs, the autophagic flux by LC3-II was quickly increased after 6 h of SM treatment and remained higher than in basal conditions up to the latest time explored (16 h, Fig. 2A). Though with a small delay, p62 levels confirmed the results obtained by LC3-II (Fig. 2A). Although apoptosis-deficient cells are known to display a more robust response to autophagic triggers [25], DKO MEFs manifested equivalent levels of autophagic flux to their WT counterparts by LC3-II and p62 analysis (Fig. 2A *vs* Fig. 2B). Likewise, levels of AO-stained orange–red AVOs in DKO MEFs confirmed its equivalency to their WT MEFs counterparts (data not shown). Next, DKO MEFs and their WT counterparts were exposed to SM before interrogating the cell cytotoxicity by PI staining. In WT MEFs, mild but distinctive levels of cell death were evident after 12 h of treatment (23.1 ± 4.1%, Fig. 2C), reaching a maximum after 24 h of continual starvation (84.1 ± 1.1%, Fig. 2C). As previously reported in HeLa cells, the pharmacological inhibition of caspases by Q-VD-OPh conferred a significant protection to WT MEFs exposed to SM (Fig. 2C). On the other hand, necrostatin-1 did not confer any protective effect (data not shown). DKO MEFs were amply refractory to cell death by SM (Fig. 2C) and remained alive even after 48 h of treatment (data not shown). To further confirm that cell death was engaging the apoptotic machinery, WT and DKO MEFs were subjected to SM and the caspase activation was assessed through time by the DEVDase activity assay (Fig. 2D). As expected, WT MEFs responded to SM by triggering an early activation of caspases (Fig. 2D). The maximum activation for WT MEFs was reported after 16 h of SM treatment while DKO MEFs exhibited no sign of caspase activation at the interrogated times (Fig. 2D). In the same line, we reported the presence of caspase-3 cleavage fragments in WT MEFs subjected to SM (Fig. 2E) whereas no fragments were seen in starved populations of DKO MEFs (data not shown). Finally, we proved that WT MEFs displayed an increase of the SubG1 fraction rescued by the addition of Q-VD-OPh (Fig. 2F). Altogether, these results were strongly evidencing that HeLa and MEF cells responded to SM by inducing autophagy and caspase-dependent cell death requiring from BAX/BAK-driven mitochondrial permeabilization.

3.3. Glucose, nutrient and growth factor starvation triggers mitochondrial outer membrane permeabilization suppressed by the pharmacological inhibition of autophagy

Our experiments were indicating that the mitochondrial outer membrane permeabilization (MOMP) was a pivotal element in the SM-driven apoptotic cell death. However, we ignored the contribution of SM-driven autophagy in the MOMP. Mitochondrial or intrinsic apoptosis is characterized by the release of pro-apoptotic proteins such as cyt C. To perform these experiments, 3-MA was used as an inhibitor of autophagy. Cellular subfractions of cytosolic soluble proteins (cytosolic) and the remaining cytosolic insoluble proteins (pellet) were obtained at short and long times of SM-treatment in the presence or absence of 3-MA. Purity of the cell subfractions was corroborated by the presence of the mitochondrial cytochrome oxidase IV (COX IV) in the pellet. Protein extracts from HeLa cells and MEFs subjected to long periods of starvation (22 and 18 h, respectively) were interrogated for the presence of cyt C in the cytosolic and pellet subfractions (Fig. 3A and B). At late times, SM-treated HeLa cells and MEFs exhibited a great translocation of cyt C to the cytosol. The comparison of these cultures in the presence or absence of 3-MA revealed no obvious differences regarding the cytosolic content of cyt C. However, if cyt C content was observed in the pellet subfraction, 3-MA was decreasing it (Pellet, Fig. 3A and B). These results were supporting that the inhibition of SM-driven autophagy was indeed increasing the release of cyt C. Alternatively, we assessed the levels of cyt C using protein extracts of HeLa cells and MEFs subjected to short

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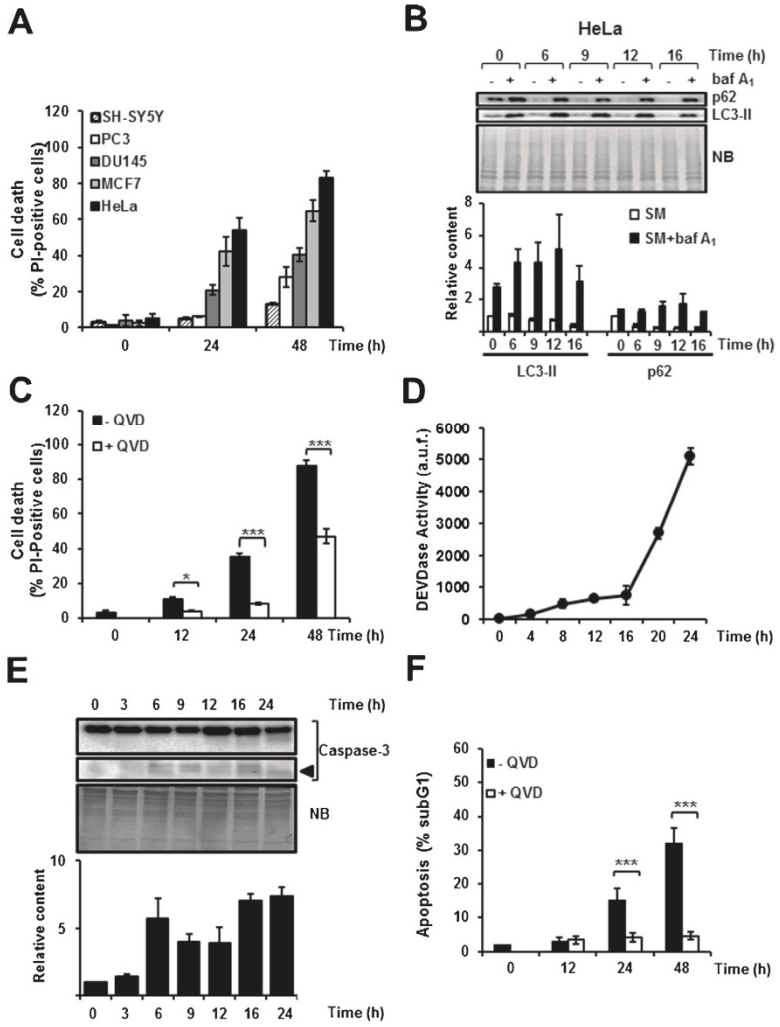

Fig. 1. Severe starvation upregulates autophagic markers and triggers caspase-dependent cell death. (A) Tumor-derived cell lines were maintained in starvation media (SM) for the times indicated in the figure. Cell death was analyzed by PI incorporation. Values are expressed as mean ± SD ($n = 3$). (B) Protein extracts of HeLa cells challenged with SM for the indicated times, were analyzed by western blot. LC3-II and p62 levels in the presence (+) or absence (−) of 100 nM baf A_1 were assessed. Naphthol blue (NB) stained membrane served as a loading control. Histograms represent p62 and LC3-II "Relative content" referred to untreated cells at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (C) HeLa cells were subjected to SM for the indicated times in the presence or absence of 10 μM Q-VD-OPh (QVD). Cell death was measured as in A and expressed as mean ± SD ($n = 3$); Student's t-test $^{*}P < 0.01$, $^{***}P < 0.001$. (D) Time course of caspase activation (DEVDase activity) using HeLa cells treated with SM for the times indicated at the figure. Activity is expressed as mean ± SD ($n = 3$) and measured as arbitrary units of fluorescence (a.u.f.). (E) Processing of caspase-3 in HeLa cells challenged with SM for the indicated times, was analyzed by western blot. Black arrow indicates cleaved caspase-3. Plot below represents the processed caspase-3 "Relative content" referred to untreated cells at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (F) HeLa cells were subjected to SM (−QVD) or SM plus Q-VD-OPh (+QVD) for the indicated times. Apoptosis (indicated by sub-G1 DNA content) were measured by flow cytometry after PI staining and cell permeabilization. Values are expressed as mean ± SD ($n = 3$); Student's t-test $^{***}P < 0.001$.

periods of starvation (8 and 7 h, respectively). To our surprise, the inhibition of autophagy by 3-MA triggered a reduction of the cytosolic cyt C. This phenomenon correlated with an increase of its content in the pellet subfraction (Fig. 3A and B). Therefore, SM-driven autophagy was triggering the release of cyt C from the mitochondria and the inhibition of autophagy was preventing it. Executioner caspases are terminal proteases in charge of dismantling the cellular physiology during the apoptotic pathway. Release of cyt C from mitochondria is an upstream event in the activation of caspase-3/7. Therefore, we surmised that the suppression of autophagy would limit the activity of caspase-3/7. As predicted, Hela cells and MEFs subjected to SM in the presence of the autophagy inhibitor 3-MA, displayed lower levels of caspase activity (Fig. 3C and D, respectively). In WT MEFs, this

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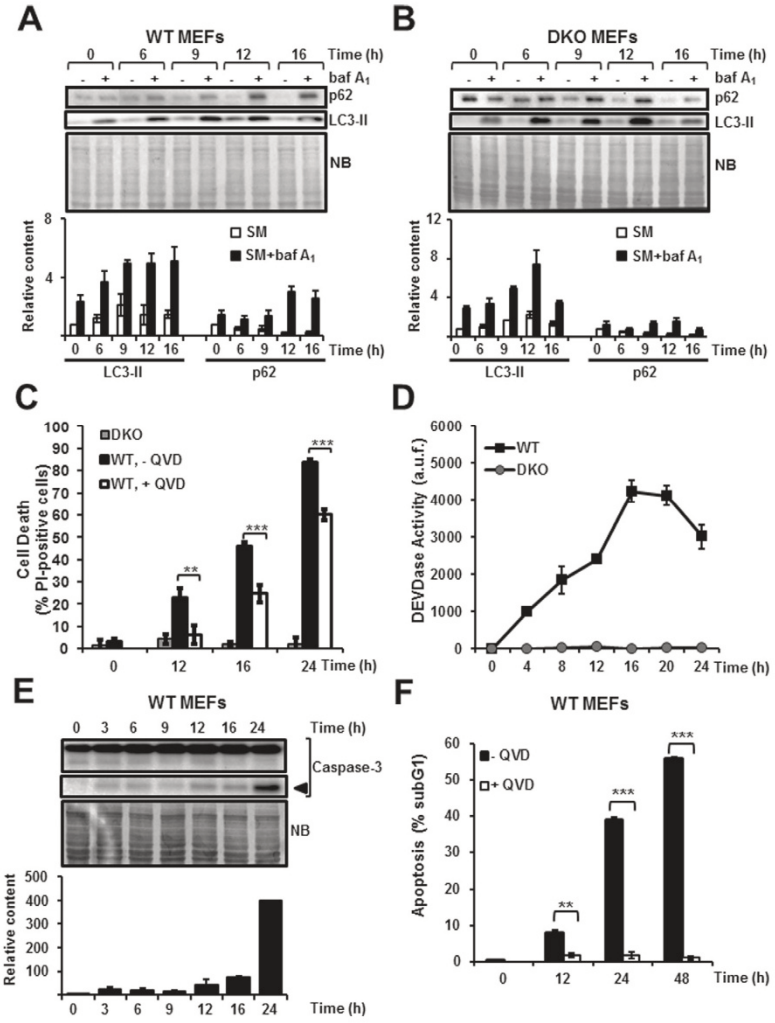

Fig. 2. Severe starvation leads to mitochondrial-dependent apoptosis. (A) Protein extracts from WT MEFs and (B) DKO MEFs subjected to SM for the indicated times, were analyzed by western blot. LC3-II and p62 levels in the presence (+) or absence (−) of 100 nM baf A_1 were assessed. Naphthol blue (NB) stained membrane served as a loading control. Histograms represent p62 and LC3-II "Relative content" referred to untreated cells at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (C) DKO MEFs and WT MEFs were challenged with SM in the presence or absence of 50 μM Q-VD-OPh for the time indicated. Cell death was analyzed by PI incorporation. Data are expressed as mean ± SD ($n = 3$); Student's t-test $^{**}P < 0.005$, $^{***}P < 0.001$. (D) WT and DKO MEFs were treated with SM for the indicated times and caspase activation (DEVDase activity) was determined at time points indicated. Activity is measured in arbitrary units of fluorescence (a.u.f.). Data are expressed as mean ± SD ($n = 3$). (E) Processing of caspase-3 in WT MEFs challenged with SM for the indicated times, was analyzed by western blot. Black arrow indicates cleaved caspase-3. Plot below represents the processed caspase-3 "Relative content" referred to untreated cells at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (F) WT MEFs were subjected to SM (−QVD) or SM plus Q-VD-OPh (+QVD) for the indicated times. Apoptosis (indicated by subG1 DNA content) were measured by flow cytometry after PI staining and cell permeabilization. Values are expressed as mean ± SD ($n = 3$); Student's t-test $^{**}P < 0.005$, $^{***}P < 0.001$.

trend was maintained up to late times, when the caspase activation of cultures in the presence or absence of 3-MA reached equivalent levels (Fig. 3D).

Owing to the unspecific effects of drugs such as 3-MA, we thought of using an alternative method to block the nucleation step of autophagy. BECLIN-1 associates to PtdInsKC3, becoming a crucial protein for the induction of canonical autophagy and a common target of inhibitory genetic strategies. To reach the greatest diminution of BECLIN-1 protein, MEFs subjected to full growth medium underwent two rounds of transfection with a *Beclin*-1 siRNA (siBecn1) or a control siRNA (siC) over a 4 days time period. These conditions resulted in a dramatic reduction of BECLIN-1 protein (Fig. 3E) and the blockage of SM-driven autophagy, as shown by the absence of p62 reduction after 6 h

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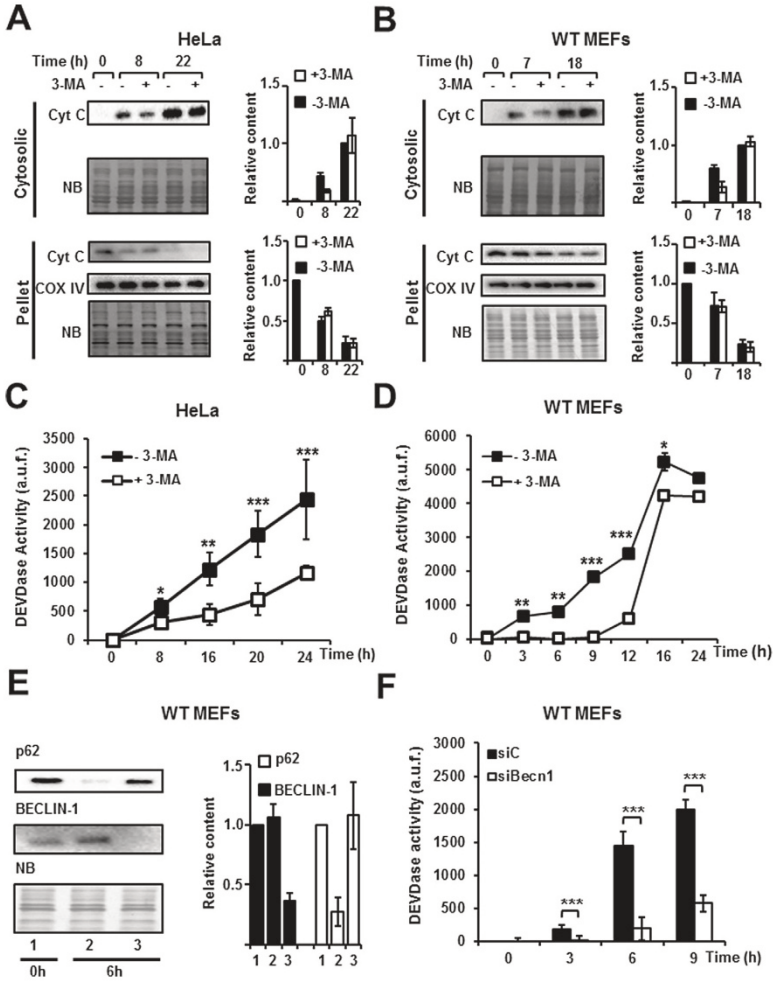

Fig. 3. SM-driven autophagy triggers early permeabilization of mitochondria and caspase activation. (A) HeLa cells and (B) WT MEFs were treated with SM in the absence or presence of 10 mM 3-MA for the indicated times. Following a complex sub-fractioning protocol, cytosolic and pellet subfractions were analyzed by western blot. Bands immunoreactive with anti-cyt C and anti-COX IV antibodies are shown. Naphthol blue (NB) stained membrane served as a loading control. Histograms represent cyt C "Relative content". In the cytosolic subfraction, the unit value of reference is the amount of cyt C released by cells at the longest time of starvation in the absence of 3-MA. In the pellet subfraction, the unit value of reference is the amount of cyt C in mitochondria of untreated cells at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (C) HeLa cells and (D) WT MEFs were treated with SM in the presence or absence of 10 mM 3-MA and caspase activity (DEVDase activity) was quantified through time. Activity is measured in arbitrary units of fluorescence (a.u.f.); Student's *t*-test $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$. (E) WT MEFs subjected to two rounds of transfection with an siRNA against *Beclin-1* (siBecn1, #3) or an siRNA control (siC, #2) were challenged with SM for the indicated times. As a control, untransfected WT MEFs (#1) are shown. Whole protein extracts were processed for western blot and analyzed with anti-p62 and anti-BECLIN1 antibodies. Naphthol blue (NB) stained membrane was used as a loading control. Histograms represent p62 and BECLIN-1 "Relative content" referred to untreated cells at time 0 (#1). Relative content is expressed as mean ± SEM of at least 3 independent experiments. (F) Time course of caspase activation (DEVDase activity) using WT MEFs transfected with siBecn1 or siC and treated with SM. Activity is measured in arbitrary units of fluorescence (a.u.f.) and data are expressed as mean ± SD of two independent experiments with 3 independent measurements per condition; Student's *t*-test $^{***}P < 0.001$.

of starvation (Fig. 3E). In regard to the activation of caspases, MEFs transfected with a siC and subjected to SM presented greater levels of active caspases than MEFs with reduced levels of BECLIN-1 (Fig. 3F). These results were evidencing, by a non-pharmacological approach, that canonical autophagy was indeed regulating SM-driven apoptotic cell death.

3.4. Glucose, nutrient and growth factor starvation requires Atg5-dependent canonical autophagy to prompt an early permeabilization of mitochondria

Canonical autophagy relies on proteins such as ATG5 for the elongation and closure of the autophagosomes and consequently,

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the *Atg5* Tet-Off MEFs m5-7 is an inestimable tool to study it. This cell line was generated from *Atg5*-/- MEFs and offers the possibility of transiently knocking-down an exogenous form of Atg5 by a 4 days doxycycline (Dox) treatment [2]. We first assessed the autophagic flux of *Atg5* Tet-Off MEFs subpopulations maintained or not in Dox over a 16 h period. As expected, the presence of Dox abrogated the autophagic flux detected by LC3-II and p62 levels (Fig. 4A). The study of cyt C localization evidenced that at long times of treatment, the Dox-treated population of *Atg5* Tet-Off MEFs released greater levels of cyt C to the cytosol than the populations kept in media without Dox (16 h, Fig. 4B). A concomitant decrease of cyt C in the pellet subfractions was reported (Fig. 4B). These findings were compatible with autophagy having a protective effect over the mitochondrial permeabilization. Remarkably, we found that at short times of treatment, this behavior was again reversed. Cytosolic extracts from Dox-treated *Atg5* Tet-Off MEFs exhibited lower cytosolic levels of cyt C than *Atg5* Tet-Off MEF not exposed to Dox (4 h, Fig. 4B). Accordingly, for the first 16 h of SM treatment, the activity of executioner caspases was significantly lower in Dox-treated *Atg5* Tet-Off MEFs than in cultures not exposed to Dox (Fig. 4C). Altogether, these findings were corroborating that, at short times, canonical autophagy (ATG5-, BECLIN-1- and PtdInsKC3-dependent) exhibited a relevant activity promoting mitochondrial permeabilization and caspase activation.

3.5. SM-driven autophagy triggers time-dependent opposite effects on cell death

In an attempt to clarify the effects of these time-depending events on the final fate of cultures, cell cytotoxicity was monitored from 12 h to 24 h in the presence or absence of 3-MA. MEFs are characterized by completing cell death in response to SM in a briefer time frame than HeLa cells (Fig. 1C compared to Fig. 2C). Owing to this behavior, they were selected for these studies. At 12 h, SM-mediated cell cytoxicity was prevented by the inhibition

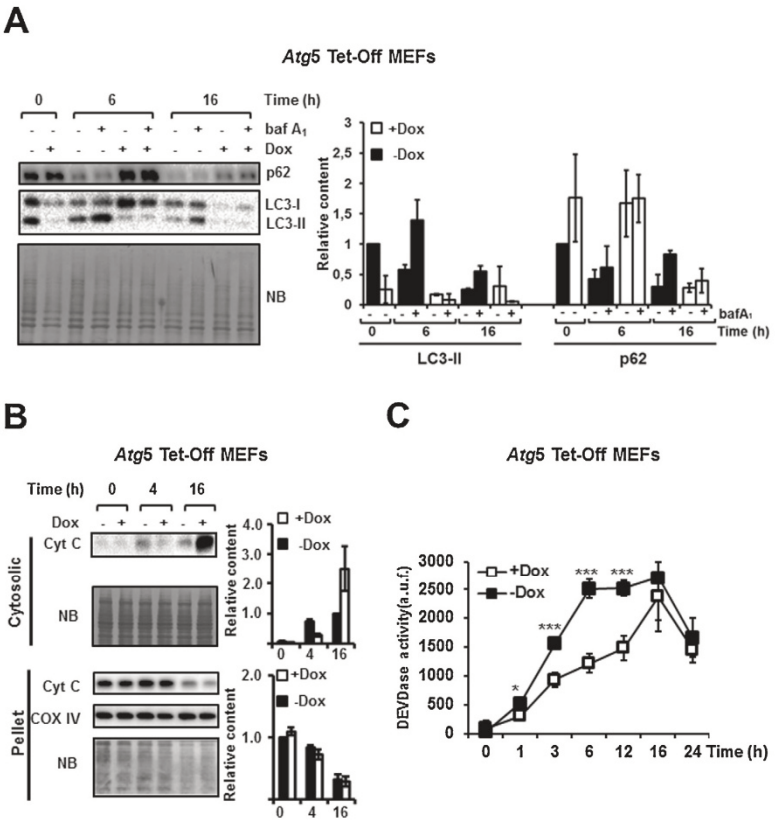

Fig. 4. SM-driven ATG5-dependent autophagy triggers early permeabilization of mitochondria and caspase activation. (A–C) *Atg5* Tet-Off MEFs in the absence (+Dox) or presence of ATG5 (−Dox) were challenged with SM for the stated times. When indicated, 100 nM baf A_1 was added for the last 3 h of treatment. (A) Protein extracts were analyzed by western blot using anti-LC3 and anti-p62 antibodies. Naphthol blue (NB) stained membrane served as a loading control. Histograms represent p62 and LC3-II "Relative content" referred to untreated cells in the presence of ATG5 (−Dox) at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (B) Following a subfractioning protocol, cytosolic and pellet subfractions were analyzed by western blot. Bands immunoreactive with anti-cyt C and anti-COX IV antibodies are shown. Naphthol blue (NB) stained membrane served as a loading control. Histograms represent cyt C "Relative content". In the cytosolic subfraction, the unit value of reference is the amount of cyt C released by cells in the presence of ATG5 (−Dox) at the longest time of starvation. In the pellet subfraction, the unit value of reference is the amount of cyt C in mitochondria of untreated cells in the presence of ATG5 (−Dox) at time 0. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (C) Time course of caspase activity (DEVDase activity). Activity is measured in arbitrary units of fluorescence (a.u.f.). Data are expressed as mean ± SD ($n = 3$); Student's *t*-test $^{**}P < 0.05$, $^{***}P < 0.001$.

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of autophagy (Fig. 5A). However, starting at 16 h, the presence of 3-MA enhanced cell toxicity in response to SM (46 ± 2.47 *vs* 78.65 ± 1.4, Fig. 5A). Since 3-MA is reported to promote autophagy in nutrient-rich conditions [26], its inhibitory activity under starved conditions was reassessed. As shown in Fig. 5B, 3-MA was able to significantly block the p62 decrease, indicating autophagy was impeded. Once the shift had been clearly established in WT MEFs, we attempted to demonstrate the reproducibility of this phenomenon in the remaining cell lines. First, for each cell line we selected an early time, where caspase activity was reduced after inhibiting autophagy and, a later time, where caspase activity was independent of the autophagic status. A growing body of evidence supports that the ability of autophagy to inhibit or promote apoptosis is highly cell line- and context-dependent [27,28]. However, at early times, inhibition of autophagy provoked a reduction of the cell death in all the cell lines tested irrespective of the inhibitory approach used (Fig. 5C). On the contrary, as previously reported by other groups [1,29], the continuous suppression of autophagy for long periods of time (16 h or longer for the studied cell lines) maximized the cell cytotoxicity in response to SM (Fig. 5D). Notably, the complete protection DKO MEFs exposed to SM in the presence or absence of 3-MA for 24 h (4.9 ± 2.6% *vs* 7.9 ± 1.2%, Fig. 5C) was indicating that, after inhibiting autophagy, cell death also required the mitochondrial permeabilization *via* BAX/BAK. These results were reaffirming the previously established role of autophagy in survival of long-term starved cell cultures but, interestingly, they were also highlighting a role of autophagy promoting early forms of caspase-dependent BAX/BAK-dependent apoptotic cell death.

3.6. Short times of SM-driven autophagy potentiates caspase-dependent apoptosis of several anticancer agents

Insufficiently irrigated solid tumors naturally undergo autophagy in response to transient periods of starvation [18]. We speculated about the contribution of autophagy to the cell lethality facing a series of antitumor agents (data not shown). Among them, we selected the compounds that were eliciting ≥20% of PI positive cells in the presence of SM for a 10 h period of time, precisely etoposide (Eto) and camptothecin (CPT). In addition, we included staurosporine (STP) as a standard inducer of intrinsic apoptosis that was also triggering ≥20% cell death at 10 h in SM. Short

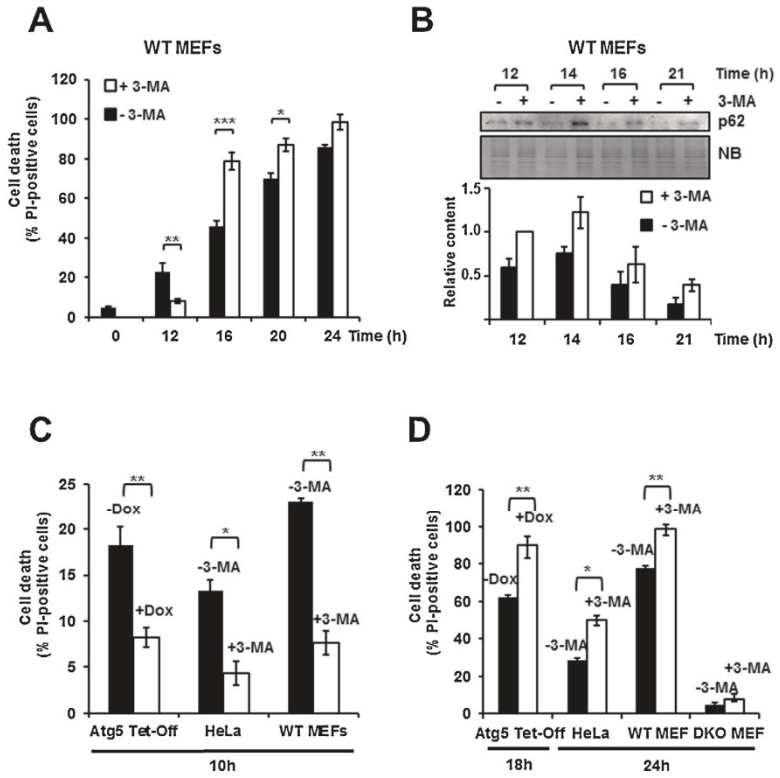

Fig. 5. SM-driven autophagy triggers time-dependent opposing effects on cell death. (A) WT MEFs were challenged with SM over a 24 h period in the presence or absence of 10 mM 3-MA. A time course of cell death was obtained by PI incorporation. Values are expressed as mean ± SD ($n = 3$); Student's *t*-test $^{*}P < 0.01$, $^{**}P < 0.005$, $^{***}P < 0.001$. (B) Whole cell extracts from WT MEFs subjected to SM in the presence or absence of 10 mM 3-MA were analyzed by western blot with a p62/SQSTM1 antibody. Naphthol blue (NB) stained membrane was used as a loading control. Plot represents p62 "Relative content" referred to 3-MA-treated cells after a 12 h period of starvation. Relative content is expressed as mean ± SEM of at least 3 independent experiments. (C and D) *Atg5* Tet-Off MEFs in the absence (+Dox) or presence of ATG5 (−Dox) were subjected to SM. In parallel, HeLa cells and WT MEFs were subjected to SM in the presence or absence of 3-MA. (B) Cell death at 10 h of starvation was measured as in (A). Values are expressed as mean ± SD ($n = 3$); Student's *t*-test $^{*}P < 0.01$, $^{**}P < 0.005$. (C) Cell death at 18 h (*Atg5* Tet-Off MEFs) or 24 h of starvation (WT MEFs and HeLa) was measured as in (A). In addition, cell death of DKO MEFs starved for 24 h in the presence or absence of 10 mM 3-MA was plotted. Values are expressed as mean ± SD ($n = 3$); Student's *t*-test $^{*}P < 0.01$, $^{**}P < 0.005$.

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starvation periods greatly increased the sensitivity of cultures to all the assayed pro-apoptotic drugs (Fig. 6A), supporting that severe starvation primes cells to die by caspase-dependent mechanisms. Then, MEFs maintained in SM in the presence or absence of 3-MA were further treated with 0.150 μM STP, 50 μM Eto and 10 μM CPT for 10 h before assessing cell death by PI staining. In each case, cell death was significantly inhibited whenever autophagy was blocked by 3-MA (Fig. 6C). Similarly, the addition of Q-VD-OPh prevented the pharmacologically-elicited apoptotic cell death of the starved cultures (Fig. 6C). In most cases, the protection conferred by the general inhibition of caspases was equivalent to the one provided by the suppression of autophagy. These findings were supported by the caspase activity assays. Whenever MEFs were impeded to undergo autophagy (Fig. 6E), reduced levels of caspase activation were found, even in the presence of the anticancer drugs. To discard an unspecific effect of 3-MA on caspase activation, we reproduced these results in the *Atg5* Tet-Off MEFs. We observed that the simple introduction of cells in SM, primed them to undergo caspase-dependent apoptotic cell death in response to Eto, STP and CPT (Fig 6B). The suppression of ATG5 reduced the sensitivity of these cultures for engaging into apoptotic cell death (Fig. 6D). Cell demise was prevented by the general inhibitor of caspases Q-VD-OPh (Fig. 6D), proving again that we were facing a caspase-dependent apoptotic cell death subroutine. To further reassert these findings, the caspase activity of cultures under the former conditions was evaluated by DEVDase

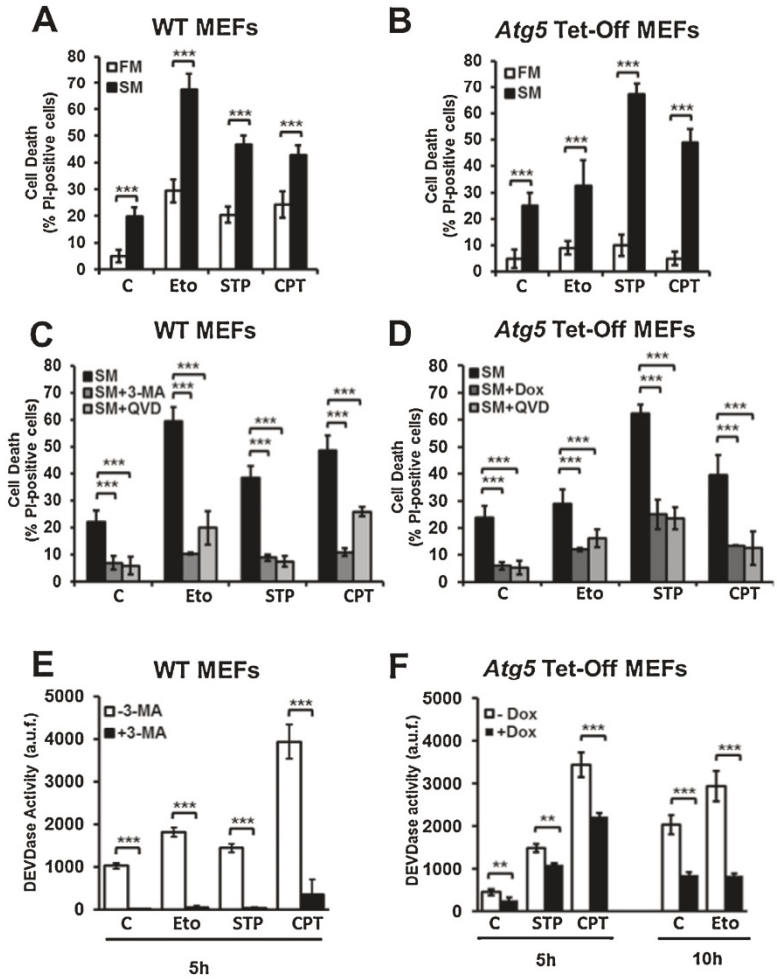

Fig. 6. SM-driven canonical autophagy sensitizes to apoptosis by several anticancer agents. (A) WT MEFs and (B) *Atg5* Tet-Off MEFs in the absence of Dox were maintained in full growth medium (FM) or starvation-medium (SM) and treated with vehicle (C, DMSO), staurosporine (STP, 0.250 μM), camptothecin (CPT, 10 μM) or etoposide (Eto, 50 μM) for 10 h. Cell death was measured by PI incorporation. Values are expressed as mean ± SD ($n = 3$). (C) WT MEFs and (D) *Atg5* Tet-Off MEFs in SM were further subjected to treatments with 3-MA, Q-VD-OPh (QVD) and Dox as indicated. Then, they were subjected to a treatment with the same drugs used in (A). Cell death was obtained by PI incorporation. Values are expressed as mean ± SD ($n = 3$) Student's *t*-test $^{**}P < 0.005$, $^{***}P < 0.001$. (E) WT MEFs in the presence or absence of 3-MA. (F) *Atg5* Tet-Off MEFs in the presence or absence of Dox were further treated with vehicle, STP, CPT or Eto for the times indicated in the figures. Caspase activation (DEVDase activity) was determined. Activity is measured in arbitrary units of fluorescence (a.u.f.) and expressed as mean ± SD ($n = 3$); Student's *t*-test $^{**}P < 0.005$, $^{***}P < 0.001$.

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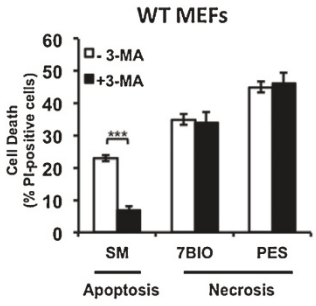

Fig. 7. Short times of starvation do not increase cytotoxicity of WT MEFs in response to necrotic inducers. WT MEFs exposed to SM (Apoptosis in the figure) were further challenged for 10 h with 25 μM 7BIO and 20 μM PES (Necrosis in the figure) in the presence or absence of 10 mM 3-MA. Cell death was then measured by flow cytometry and the count of PI-stained cells. Values are expressed as mean ± SD of two independent experiments with 3 independent measurements per condition.

activity assay. Autophagy deficient subpopulations of *Atg5* Tet-Off MEFs (Fig. 6F) exhibited reduced levels of caspase activity in SM when compared to the same cells undergoing autophagy. Addition of STP, CPT and Eto to the deprived medium increased the caspase activity in comparison to the levels reached in SM by itself ("C", Fig. 6F). Nonetheless, the autophagy deficient subpopulations reached significantly lower levels of caspase activity for each of the used drugs (Fig. 6F). Next, we wondered whether the lethal actions of autophagy would be restricted to the context of apoptotic drugs or, on the contrary, would be also evident when applying necrotic triggers. To perform these experiments, 7-bromo-indirubine-3-oxime [30] (7BIO) and 2-phenylethynesulfonamide [31] (PES), two caspase-independent necrotic inducers were employed. Remarkably, WT MEFs exposed to SM in the presence of 3-MA were as sensitive to the lethal effects of 7BIO and PES as the autophagic-competent cells (Fig. 7). We concluded that 3-MA protective actions were a singularity of caspase-dependent apoptotic inducers. Altogether, these results were strongly proving that in the context of starvation, autophagy was accelerating the naturally occurring or pharmacologically-imparted caspase-dependent apoptotic cell death.

4. Discussion

Severe forms of starvation are reported to elicit both autophagy and cell death [1,12]. The interplay between autophagy and cell death processes is a matter of controversy. Here we show how nutrient, glucose and growth factor deprivation elicit cell death in a variety of ontogenically divergent cell lines. At short times, starvation-driven caspase-dependent apoptosis relies on the machinery of autophagy and requires the caspase activation and the mitochondrial permeabilization *via* BAX/BAK. The ongoing SM-driven autophagy relies on ATG5, BECLIN-1 and the activation of PtdInsKC3, as proven by 3-MA. On the contrary, at longer times of starvation, the cell death is characterized by its independency from the autophagy machinery. Moreover, at this time, the inhibition of autophagy is promoting cell survival. When focusing on short-term starvation, autophagy-driven caspase-dependent cell death is enhancing the apoptotic cell death in response to several pro-apoptotic antitumor drugs. Accordingly, either the inhibition of autophagy or the blockage of executioner caspases, translate into a strong protection facing these antitumor drugs.

Growing tumors are naturally exposed to periods of insufficient or null irrigation [4,18]. Similarly, ischemia is a pathological situation characterized by a sudden interruption of blood supply [12]. These cellular contexts share a common trait, a shortage of oxygen, nutrients, glucose and trophic factors. Several approaches have intended to mimic these environments *in vitro* by combining the depletion of single amino acids, glucose, growth factors, oxygen and serum. Starvation is a powerful signal to activate macroautophagy, a bulk form of autophagy, triggered to cope with the energetic and metabolic demands of a deprived cell. During the completion of autophagy, the lysosome-mediated degradation of cytoplasmic material will supply cells with energy and metabolites that cannot be synthesized *de novo*. Moreover, anaplerotic reactions taking place during starvation, will sustain the necessary biosynthesis of proteins and nucleic acids. In addition, cataplerotic pathways will efficiently intertwine the metabolic intermediates as needed. Sufficient levels of energy will be obtained, for instance, through cataplerotic reactions and the beta-oxidation of fatty acids. Therefore, privation of one single nutrient, sugar or lipid can create a physiological compensation that can alter the real effect of starvation. Our work was performed with a medium depleted of all these components to avoid any kind of artifact. It is worth to mention the controversy over the role of glucose withdrawal in the induction of autophagy [19,32,33]. Interestingly, a recent publication shows that the simple glucose withdrawal triggers cell death by either apoptosis or necrosis without an apparent induction of the autophagic flux [19]. However, we proved that the composition of our buffer and, specifically, the absence of glucose, did not impede a series of ontogenically divergent cell lines to undergo autophagy (Figs. 1 B and 2 A and B). In agreement with their findings, we observed that the reintroduction of glucose into our complete starvation media (SM) slightly increased the autophagic flux of WT MEFs (data not shown), thus supporting glucose is positively regulating the autophagic flux.

Here, we provide evidence that mitochondria play a seminal role in the clearance of starved mammalian cells. In agreement with our data, interleukine-3 (IL-3) withdrawal evidenced the relevance of BAX/BAK-regulated mitochondrial homeostasis in the apoptotic demise of IL-3 dependent bone marrow-derived cells [1]. Similarly, primary neonatal rat cardiac myocites under glucose and serum withdrawal were cleared through the mitochondrial apoptotic pathway of cell demise [12]. Interestingly, the pharmacological inhibition of caspases with zVAD-fmk prevented the terminal features of apoptosis without restoring the cellular morphology and the contractile capability of these myocites, thus suggesting that energy depletion and caspase-independent events might be also important players in this process. In line with these findings, the general inhibition of caspases is not fully protecting HeLa and WT MEFs from SM-driven cell death. For instance, cell cytotoxicity in HeLa cells (48 h) and WT MEFs (24 h) subjected to SM in the presence of Q-VD-OPh is 47 ± 4.5% and 61 ± 5.2%, (Figs. 1 C and 2 C, respectively). In addition, only BAX/BAK deficient cells become refractory to long-term SM-driven cell death (Fig. 2C). It would then appear that the early induction of intrinsic apoptosis would lead starved cells into cell death. In this sense, we have observed an early release of AIF from mitochondria (data not shown), which added to the release of other pro-death proteins from this organelle, would justify why BAX/BAK deficient MEFs surpass Q-VD-OPh in their protecting capacity. Others have reported that the type of cell death subroutine engaged in response to starvation is cell-line dependent rather than stimulus-dependent. For example, under glucose starvation Rh4 rhabdomyosarcoma cells undergo necrotic cell death while HeLa cells and MEF, apoptotic cell death [19]. We cannot predict whether all the cell lines under SM would be consistently undertaking the same subroutine of cell death. In this sense, density could be playing a determinant role in the type of cell death, necrotic or apoptotic, in response to serum withdrawal [34].

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Therefore we cannot rule out if changes in the 90% density, the standard cell confluency of our experiments, will alter the type of cell death.

Despite the early anti-apoptotic effects achieved through inhibition of autophagy, we demonstrate that long-term starved cultures, unable to undergo autophagy, exhibit a greater cytotoxicity. Thus indicating that after long times of starvation, autophagy acts as a protective mechanism. To perform these experiments, autophagy was suppressed for a long period of time and thus, one possible explanation for the increased death is the inefficiency of our approaches to block autophagy. This hypothesis is especially interesting in the context of a chemical inhibitor such as 3-MA. This drug presents opposite effects regulating autophagy under nutrient-rich (promotion) and nutrient-poor (inhibition) conditions caused by disparities in the kinetics of class I and III PtdIns 3-kinase inhibition [26]. Owing to these discrepancies, the inhibitory effects of 3-MA at long times of starvation were confirmed by analyzing the levels of p62 by western blot (Fig. 5B) and AO-positive vesicles by fluorescent microscopy (data not shown). In addition to the genetic strategies employed (siBecn1 and ATG5 deficient cells), we thought of using a second chemical inhibitor of autophagy, spautin-1 [35]. Unfortunately, while spautin-1 was able to block the caspase activation at short times of starvation, it presented a greater lethal profile than 3-MA when maintained for long times in starved cultures (results not shown). As a second hypothesis, we propose the activation of a second wave of ATG5-, Beclin-1- and Vps34-independent form of non-canonical autophagy. However, the preservation of p62 content and the diminished levels of AO-positive vesicles by 3-MA argue against this idea (data not shown). The third hypothesis has to do with mitochondria and energy. Only the absence of BAX/BAK confers a full protection (up to 48 h) facing the inhibition of autophagy, pointing out to a prominent role of the mitochondrion or mitochondrial-related phenomena in the induction of cell death. In this sense, a time-dependent decrease in the energy production is a natural response to the glucose withdrawal and ensuing glycolytic production of ATP. Under these circumstances, the beta-oxidation of fatty acids and the respiration of metabolic intermediates originated after the degradation of cytoplasmic material within the autophagolysosomes, might be partially compensating the shortage of ATP. However, the inhibition of autophagy might be diminishing these alternative pathways of energy production. In this sense, autophagy is reported to act as a protective mechanism against bioenergetic catastrophe [36]. It is then possible to hypothesize that, either the exhaustion of ATP resources or the decrease of ATP levels under a hypothetical threshold, might be playing a relevant role in the death ensuing SM-driven autophagy inhibition. To illustrate this situation, we find the case of immortalized interleukin-3 (IL-3) dependent bone marrow cell lines from *Bax-/-Bak-/-* mice, which are protected from apoptosis in response to IL-3 withdrawal. In this settings, inhibition of autophagy by 3-MA or chloroquine is eliciting cell death. However, the supplementation of a permeable form of pyruvate intended to produce NADH and ATP restores the viability of these cells [1].

An expanding body of evidence sustains that autophagy is governing apoptotic cell death under specific developmental circumstances. For example, it is known that autophagy drives caspase-dependent and independent cell death necessary for the regression of *Drosophila melanogaster* salivary glands [37]. However, the truth is that *Atg5*,*Atg7* and *Beclin-1* knockout mice do not present deficiencies in the developmental forms of programmed cell death [38]. Notwithstanding these discrepancies, multiple findings support that, in adult cells, autophagy does regulate other subroutines of cell death under context-specific circumstances. Putting aside those models where autophagy seems to be by itself the ongoing subroutine of death, all gathered into the common term "autophagic cell death", it is noteworthy the existence of autophagy-dependent apoptotic cell death models [39–46]. Our findings fall into this last scenario since, at short times of starvation, autophagy is regulating apoptotic cell death. For example, overexpression of ATG1 in fly exacerbates autophagy, resulting in increased apoptosis [39]. In rat striatal neurons, overstimulation of kainic acid (KA) receptors activates autophagy-driven intrinsic apoptosis, thus placing autophagy upstream of KA-driven excitotoxicity [40]. A similar case is found in cortical neurons since STP-elicited autophagy is driving the apoptotic cell death [41]. Similarly, clearance of lymphoblastic leukemia cells exposed to the glucocorticoid dexamethasone requires the induction of autophagy prior to the mitochondrial apoptotic death [42]. A similar response is observed in multiple myeloma cells treated with dexamethasone [43]. Inhibition of autophagy prevents the apoptosis ensuing TNFα treatment of sarcoma cells with an inhibited NF-kB pathway [44], ER stress in non-transformed fibroblast [45], and serum and potassium deprivation of cerebellar granule cells [46]. Unfortunately, there is no consensus on the precise mechanism intertwining autophagy and apoptosis. A growing number of distinct pathways with key proteins are being proposed [13,17,47–52]. For example, one of these proteins is FoxO1. The protein by itself and its localization are key factors in determining the induction of autophagic and apoptotic cell death in HCT116 and HeLa cells subjected to serum starvation or hydrogen peroxide [47]. Yet another protein that links the autophagic and apoptotic phenomena is ATG5. The calpain-dependent cleavage of ATG5 into a 24-kDa cleavage fragment uncovers a "BH3-only" function of this protein [48]. Notably, these findings are in agreement with calpain being required for the induction of macroautophagy in mammalian cells [49]. Although in our experimental paradigms the suppression of ATG5 reduces the short-term SM-driven apoptosis, in the same settings, the inhibition of calpain by the pharmacological inhibitor calpeptin did not confer any protection facing SM (data not shown). Thus, we don't favor calpain and ATG5 cleavage as key regulators in the induction of autophagy-driven apoptosis by SM. Interestingly, ATG5 is also a validated link between autophagy and the extrinsic pathway of apoptosis. ATG5 forms a complex with the death receptor adaptor protein FAS-associated death domain (FADD) and caspase-8, bringing this complex in proximity to p62/SQSTM1. These three proteins participate in the activation of caspase-8 and the ensuing apoptotic cell death [50]. Another possible mechanism to link autophagy and apoptosis is the covalent assembly of the ATG12-ATG3 complex occurring during starvation. While the proper assembly of this complex triggers intrinsic apoptotic cell death, its disruption elicits the enlargement of the mitochondrial mass and a concomitant increase of the anti-apoptotic Bcl-2 proteins [51]. Alternatively, HCT116 colon cancer cells subjected to nutrient-serum starvation display a concomitant upregulation of PUMA and p21. While p21 presents unknown protective effects at the mitochondrial level, PUMA is in charge of eliciting the intrinsic apoptosis [13]. Yet in another system, the simple overexpression of PUMA or BAX causes a form of selective mitophagy accompanied by cyt C release and apoptotic cell death. In these experimental paradigms, down-regulation of several ATGs has protective effects facing apoptosis, suggesting that autophagy contributes to the apoptotic response [17]. Moreover, in support of our findings, the same group concluded that autophagy might be contributing to the efficient release of cyt C and so, to the induction of apoptosis. Other authors propose the disturbance of an autophagy-driven epigenetic program as the key element to shift autophagy from a pro-survival to a pro-death mechanism. Namely, the down-regulation of the histone acetyltransferase hMOF, the ensuing reduction in H4K16 acetylation level and the

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transcriptional regulation of autophagy-related genes are steps necessary for the proper progression of autophagy and its pro-survival effects [52]. We ignore the relevance of each of these pathways in our experimental paradigm. We propose future high throughput studies to address this issue.

Our work underscores the paramount role of autophagy inducing caspase-dependent apoptotic cell death in short-term starved cells and, under the same circumstances, enhancing the caspase-dependent apoptotic cell death in response to a series of apoptotic stressors. In this sense, autophagic cells in core areas of multicellular spheroids, which mimic the three dimension structure of a tumor, undergo glucose shortage, which make them particularly sensitive to the mitochondrial dysfunction [36]. In our studies, we used a series of apoptotic drugs that are known to elicit the intrinsic apoptotic pathway and hence, that trigger the mitochondrial failure. We observe that, in comparison to the same cells in full media, a short-term starvation is strongly favoring the apoptotic cell demise (Fig. 6A and D). In the same line, HCT116 cells up-regulating PUMA in response to nutrient-serum starvation, display an increased sensitivity to the Bcl-X_L inhibitors [13]. Alternatively, the abrogation of ATG3-ATG12 complex protects cells from mitochondrial pro-apoptotic triggers [51]. On the opposite, our results prove that cell death in response to drugs that don't require the BAX/BAK pro-apoptotic gateway (7BIO and PES, Fig. 7), remains unaffected by starvation.

In conclusion, our manuscript introduces the first model in cell culture characterized by time being the only determinant of the modulation of apoptosis by autophagy. The shift from apoptosis promotion to inhibition has been circumscribed temporally, thus simplifying the research of molecular events governing it. Moreover, the shift has translational implications because it becomes a crucial factor determining the outcome of chemotherapy in solid tumors. In addition, it remarks the heterogeneity present in a tumor mass, not only of genotypic or phenotypic nature but chrono-biological as well. In other words, concerning cell death mechanisms, time is a coordinate to be taken into account in the starvation conditions imposed by the solid tumor biology.

Conflict of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The author's thanks Dr. P. Codogno for the *Atg5* Tet-Off MEFs m5-7 (originated at Prof. Mizushima's laboratory) and Dr. S.E. Lupold for the PC3 and DU145 cell lines. This work was supported by Ministerio de Economía y Competitividad SAF2011-29730. VJY is under a "Retention of Research Talent" contract of "Programa Banco de Santander". PM was supported by a fellowship (AH9815758) from AGAUR (Generalitat de Catalunya). University of Lleida Funds (Ajuts Pont 2015) contributed to the completion of this work.

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2 2-Phenylethynesulfonamide (PES) uncovers a necrotic process regulated by oxidative stress and p53

Biochemical Pharmacology 91 (2014) 301–311

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

2-Phenylethynesulfonamide (PES) uncovers a necrotic process regulated by oxidative stress and p53☆

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ARTICLE INFO

Article history:
Received 28 May 2014
Accepted 7 August 2014
Available online 16 August 2014

Chemical compounds studied in this article:
2-Phenylethynesulfonamide (Pubmed CID 327653)
L-Buthionine-sulfoximine (Pubmed CID 119565)
N-acetyl cysteine (Pubmed CID 12035)
Dithiothreitol (Pubmed CID 19001)
Necrostatin-1 (Pubmed CID 282833)
3-Methyladenine (Pubmed CID 1673)
Q-VD-OPh (Pubmed CID 11237609)

Keywords:
Anticancer drug
Necrosis
p53
Reactive oxygen species (ROS)
2-Phenylethynesulfonamide (PES)
Pifithrin-μ

ABSTRACT

2-Phenylethynesulfonamide (PES) or pifithrin-μ is a promising anticancer agent with preferential toxicity for cancer cells. The type of cell death and the molecular cascades activated by this compound are controversial. Here, we demonstrate PES elicits a caspase- and BAX/BAK-independent non-necroptotic necrotic cell death, since it is not inhibited by necrostatin-1. This process is characterized by an early generation of reactive oxygen species (ROS) resulting in p53 up-regulation. Accordingly, thiolic antioxidants protect cells from PES-induced death. Furthermore, inhibiting the natural sources of glutathione with L-buthionine-sulfoximine (BSO) strongly cooperates with PES in triggering cytotoxicity. Genetically modified p53-null or p53 knocked-down cells show resistance to PES-driven necrosis. The predominant localization of p53 in chromatin-enriched fractions added to the up-regulation of the p53-responsive gene p21, strongly suggest the involvement of a transcription-dependent p53 program. On the other hand, we report an augmented production of ROS in p53-positive cells that, added to the increased p53 content in response to PES-elicited ROS, suggests that p53 and ROS are mutually regulated in response to PES. In sum, p53 up-regulation by ROS triggers a positive feedback loop responsible of further increasing ROS production and reinforcing PES-driven non-necroptotic necrosis.

1. Introduction

PES (2-phenylethynesulfonamide), also named pifithrin-μ, was first identified as a compound able to repress apoptosis induced by the translocation of p53 to the mitochondria [1]. Further studies have revealed that PES is an effective inducer of cell death with increased selectivity for cancer cells [2]. The pharmacological actions of PES include the blockage of both the proteasome and the autophagic process [3]. These to actions converge in increasing the proteotoxic stress of the highly metabolically active cancer cells but, also, in inactivating proteins, such as p53, through its inclusion in insoluble aggregates [3]. This p53 inactivating mechanism, plus the fact that p53-defective cells are not resistant to PES, has supported the idea of a null involvement of p53 in the process of cell death triggered by this molecule. Cell death, cell cycle arrest, senescence, DNA repair, glucose metabolism, ROS generation and autophagy are some of the cellular phenomena regulated by p53 [4]. p53 is the most frequently mutated tumor suppressor gene in human cancer [5,6]. Most cellular insults trigger intracellular signaling cascades that converge in p53 activation, for example DNA damage, viral infection, oncogene activation or ROS generation [4,7]. In response to these stimuli, p53 is stabilized and engages a plethora of molecular pathways, including both transcriptional and non-transcriptional events [4,8].

☆ This work was supported by Spanish Government, MINECO (Ministerio de Economía y Competitividad), project SAF2011-29730. Paolo Mattiolo was supported by a FI fellowship – AH9815758 – from AGAUR (Generalitat de Catalunya).

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http://dx.doi.org/10.1016/j.bcp.2014.08.005
0006-2952/

Necrotic cell death was initially described as a passive mechanism of cell demise. For a while, necrosis became synonym of accidental death and was defined by its morphological traits [9]. However, further research uncovered necrosis as a regulated process in many instances [10,11]. Necroptosis is a type of regulated necrosis mediated by the activation of receptor-interacting protein kinase 1 (RIPK1) and RIPK3. Necrostatin-1 is a RIPK1 inhibitory drug, which allows the inhibition and identification of necroptotic cell death [12].

We set out to gain a better understanding of how PES kills cancer cells. PES elicited a non-apoptotic, non-autophagic, non-necroptotic cell death in SH-SY5Y and HCT116 cells. Interestingly, PES triggered an early burst on ROS content, which was required for cytotoxicity. We observed a ROS-dependent increase in the p53 protein levels, mostly located at the chromatin-enriched fraction and followed by an increase on p21 content. p53 up-regulation boosted cell sensitivity to PES-mediated necrosis. In this sense, silencing p53 diminished the cytotoxic response to PES. In sum, our results indicate that ROS produced by PES trigger the up-regulation of p53, which, in turn, increases the production of ROS, resulting in a non-necroptotic form of necrosis.

2. Materials and methods

2.1. Cell culture and drug treatments

Colon adenocarcinoma HCT116 and HCT116 p53−/− cells were obtained from Dr. Vogelstein's laboratory [13]. The immortalized MEF *Bax*−/−*Bak*−/− and their WT counterparts were obtained from Dr. Korsmeyer's laboratory [14,15]. SH-SY5Y, HEK293, U87MG, HeLa and HL-60 cell lines were obtained from the American Type Culture Collection. SH-SY5Y, HEK293, U87MG, HeLa and MEFs were maintained in DMEM, HL-60 in RPMI and all the colon-derived cells in McCoy's media. All media were supplied by Lonza (Rockland, ME, USA) and supplemented with 10% FCS (Gibco part of Invitrogen, Paisley, UK). Plasmocin™ (InvivoGen, San Diego, CA, USA) at 5 μg/ml final concentration was added as a prophylactic antibiotic. Cultures were regularly PCR-tested to confirm mycoplasma-free conditions. Cultures were maintained at 37 °C in water-saturated, 5% CO_2 atmosphere. Stock solutions of PES and the other chemicals were prepared in DMSO. From these stock solutions, drugs were delivered to the culture media and adjusted to the final concentrations reported in the text and figures. The serial dilution procedure was used in concentration-dependency determinations.

2.2. Chemical reagents

CellTiter 96® kit containing the reagent MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxy-methoxy phenyl)-2-(4-sulfophenyl)-2H-tetrazolium) was provided by Promega (Madison, WI, USA). Pierce Biotechnology (Rockford, IL, USA) provided Alamar blue™ Cell Viability Assay Reagent. Q-VD-OPh (Q-Val-Asp(non-methylated)-OPh) and 3-methyladenine (3-MA) were obtained from Calbiochem part of Merck KGaA (Darmstadt, Germany). AcDEVDafc (Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl-coumarin) was purchased from Enzo Life Sciences (Farmingdale, NY, USA). Dithiothreitol (DTT) was from Thermo Fisher Scientific (Waltham, MA, USA). Necrostatin-1 was purchased from Tocris Bioscience (Ellisville, MI, USA). Staurosporine (STS), Spautin-1, bisBenzimide (Hoechst 33342), PI (Propidium iodide), N-acetyl-L-cysteine (NAC) and L-buthionine-sulfoximine (BSO) were supplied by Sigma–Aldrich (St. Louis, MO, USA). Unless otherwise stated, the non-listed reagents were also from Sigma.

2.3. Cell survival and cell death and nuclear morphology assessment

To determine cell survival, MTS or Alamar blue reagents were used indistinctly. Both assays couple a nonspecific cellular reductase activity of viable cells to the reduction of a dye into colored (MTS) or fluorescent (Alamar blue) products, which are subsequently quantified. Percentage of viability was obtained by referring these values to the ones obtained with a population of vehicle treated cells. To assess cell death, cultures stained with PI, were trypsinized and subjected to flow cytometry analysis. Nuclear morphology was observed by direct fluorescence microscopy of cells in media containing 0.05 μg/ml bisBenzimide plus 12.5 μg/ml PI as previously described [11].

2.4. Caspase activity

Effector caspase activation (DEVDase activity) was obtained by quantifying the fluorescence released from Ac-DEVD-afc substrate after incubation at 37 °C in the lysed cell cultures. This method has been validated and described in our previous work [16].

2.5. Overexpression of p53

HCT116 p53−/− cells, plated at 1.2×10^6 in p35 wells, were transfected with 6 μg of pCMV-Neo-Bam p53 WT or pCMV-Neo-Bam [17] in combination with 0.6 μg of a plasmid containing GFP. DNA was prepared in Opti-MEM™ media (Life Technologies, Carlsbad, CA, USA) before adding Turbofect transfection reagent (Thermo Fisher Scientific, Waltham, MA, USA) as described in the instructions manual. After 24 h, cells transfected were plated in M96 plates for drug treatment and cell viability assessment.

2.6. Lentiviral production and infection

FSV and FSV p53 lentiviral constructs were gently provided by Dr. Xavier Dolcet and Dr. Núria Eritja. Vectors contained a U6 promoter for expression of short hairpin RNAs against p53 (GTCCAGATGAAGCTCCCAGAA) and the Venus variant of YFP under the control of an SV40 promoter for monitoring transduction efficiency. Lentiviral particles were produced in 293T cells co-transfected by the calcium phosphate method with the above plasmid plus plasmids coding for the envelope and the packaging systems (VSV-G and Δ8.9, respectively). The day after transfection, 293T cells were switched to media containing no anti-mitotics and left for 2–3 days. Supernatants were then harvested, filtered through a 0.45 μm filter, and directly added to cultures in the presence of Polybrene (Merck Millipore part of Merck KGaA, Darmstadt, Germany).

2.7. Electron microscopy

Cells were collected, washed twice in PBS (150 mM ClNa, 2.7 mM ClK, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and fixed for 30 min at 4 °C in 100 mM phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. After rinsing the pellets twice with PBS at 4 °C, the cells were post-fixed in buffered OsO_4, dehydrated in graded acetone and embedded in Durcupan® ACM resin (Sigma–Aldrich, St. Louis, MI, USA). Ultrathin sections mounted on copper grids were counterstained with uranyl acetate and lead citrate. A transmission electron microscope (Zeiss EM 910) was employed to visualize the cellular ultrastructure.

2.8. Intracellular ROS measurement

The quantification of intracellular ROS was based on fluorescence of the compound DCF that results from cell metabolization

and ROS action on the precursor compound 2′,7′-dichlorofluorescin diacetate. Briefly, cells were seeded in 96 well plates. Media was replaced 48 h later by Phenol-red free Hank's Balanced Salt Solution containing 10 μM DCF. After an incubation of 30 min, the reagent was removed and cells were washed with pre-warmed PBS. Cultures were then immediately incubated in full media containing the investigated drug. At the times desired, fluorescence was read at 485/530 nm (excitation/emission wavelengths) with a plate reader (Infinite® M200, Tecan, Maennedorf, Switzerland).

2.9. Protein extraction and Western blotting

To perform whole cell extracts, cells were lysed in a buffer containing 100 mM Tris/ClH pH 6.8, 1% SDS, 1 μM EDTA, plus the cocktail of protease inhibitors from Sigma followed by sonication. After a centrifugation at 12,000 × *g* for 15 min, a total protein extract was obtained. To obtain extracts from specific cell subfractions (cytosolic, nucleoplasmic and chromatin-enriched), we followed the protocol described in a previous publication [18]. The protein concentration was determined by means of the DC Protein Assay (BioRad, Hercules, CA, USA). Volumes were calculated to equalize the protein load in SDS 12%-polyacrylamide gel electrophoresis. Following electrotransfer to 0.45 μm PVDF membranes (EMD Millipore part of Merck KGaA, Darmstadt, Germany), we applied the following antibodies: anti-p53 clone BP53-12 (Upstate, part of Merck KGaA, Darmstadt, Germany), anti-p21 clone CP74 (Sigma, St. Louis, MI, USA), anti-lactate dehydrogenase (LDH) (Rockland™ Immunochemicals Inc., Boyertown, PA, USA), anti-polypyrimidine tract binding protein 1 (PTBP1) ab5642 (Abcam, Cambridge, England, UK) and anti-GAPDH-Peroxidase clone GAPDH-71.1 (Sigma, St. Louis, MI, USA). Immunoblots were finally developed with the Immobilon™ reagent from Millipore part of Merck KGaA (Darmstadt, Germany). Chemiluminiscence was recorded and densitometric analysis was performed by means of a Chemidoc™ apparatus and the Image Lab version 4.0.1 software from Bio-Rad. To quantify expression of p53, values of signal intensity were normalized to the appropriate signal in control extracts. Alternatively, to quantify p53 or p21 content in Fig. 6B or Fig. 7A, values of signal intensity were first referred to the values of GAPDH and then, normalized to a non-induced condition.

3. Results

3.1. PES triggers caspase-independent non-apoptotic cell death

A panel of human-derived cell lines (SH-SY5Y from neuroblastoma, U87MG from grade III astrocytoma, HL-60 from acute promyelocytic leukemia, HEK293 from embryonic kidney, HeLa from cervical cancer, and HCT116 from colorectal carcinoma) was used to assay the cytotoxicity induced by increasing concentrations of PES. As shown in Fig. 1A, PES triggered loss of cell viability in a concentration-dependent manner. In support of previous observations, the non-tumor derived HEK293 cells displayed a greater resistance to PES-driven toxicity [2]. We focused our research on HCT116 and SH-SY5Y, two tumor-derived cell lines with different ontogeny, being SH-SY5Y neatly more sensitive to PES than HCT116. Plasmatic membrane rupture by means of PI permeability was evaluated over time at concentrations of 12.5 and 25 μM PES in SH-SY5Y and HCT116, respectively. As shown in Fig. 1B and C, 12.5 μM PES for 24 h in SH-SY5Y was equivalent to 25 μM for 48 h in HCT116. At these concentrations, 60% of SH-SY5Y and HCT116 cells were PI positive after 24 h and 48 h, respectively. To assess whether the type of cell death was apoptotic, we

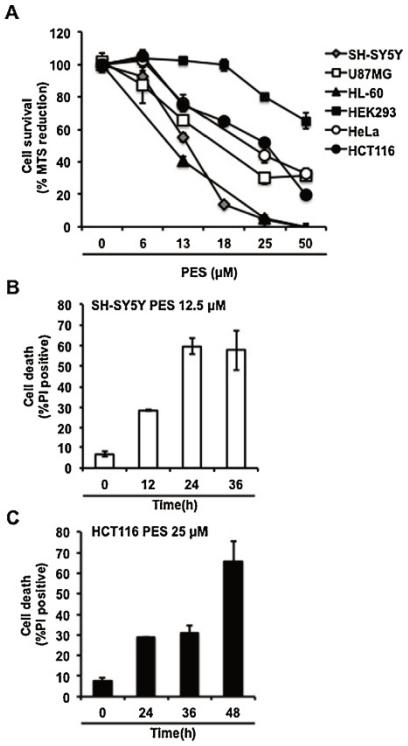

Fig. 1. PES-driven cell death is concentration and time dependent. (A) The cell lines indicated in the graph were treated for 24 h with increasing concentrations of PES. Cell viability was calculated by the MTS reduction assay. (B) SH-SY5Y and (C) HCT116 WT cells were exposed to the indicated concentrations of PES. Cell death by PI staining was quantified over time. Each value in the graphical representation is the average ± S.D. of at least 3 independent experiments with 3 independent measurements per experiment.

double stained nuclei with bisBenzimide and propidium iodide (PI). In these experiments, HCT116 and SH-SY5Y did not present evident chromatin condensation or nuclear fragmentation, which are distinctive traits of apoptosis. A similar behavior was observed in U87MG, HeLa, 293HEK and HL-60 cells when assaying concentrations of PES that at 24 h triggered death in 50% of the cell populations (results not shown). To sustain the absence of apoptotic death in HCT116 and SH-SY5Y, caspase activation (DEVDase activity) was evaluated through time. As expected, PES was unable to trigger caspase activation in both cell lines (Fig. 2A and B). As a control, we assessed caspase activity in response to staurosporine (STS), thus confirming the caspase functionality in both cell lines (Fig. 2A and B). Equivalent results were obtained when applying the same strategy to U87MG, HeLa, 293HEK and HL-60 cells (Fig. 2C). To further discard apoptosis as the predominant death process engaged by PES, we tested its effects on mouse embryonic fibroblasts (MEFs) defective in both, BAX and BAK. These proteins are key positive mediators at the mitochondrial or intrinsic pathway of apoptosis and, therefore, MEF *Bax*−/−*Bak*−/− cells become completely unresponsive to the stimuli that trigger it. As expected, MEF *Bax*−/−*Bak*−/− cells displayed resistance to STS as compared to their WT counterparts. However, deficiency of BAX and BAK provided no protection

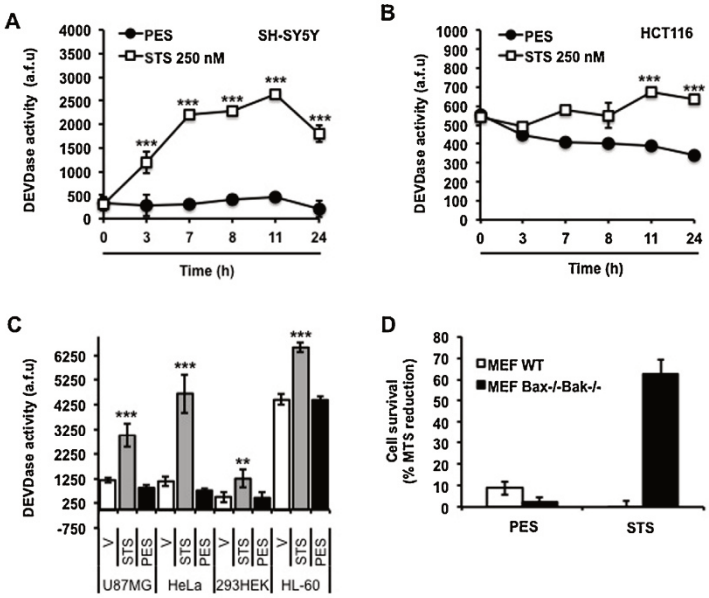

Fig. 2. PES triggers caspase-independent non-apoptotic cell death. (A) SH-SY5Y cells were challenged with 12.5 μM PES or STS 250 nM and the time course of caspase activation (DEVDase activity) was determined. Activity is measured in arbitrary fluorescence units (a.f.u). Each point is the average ± S.D. of 6 independent measurements. The plotted profile is representative of 3 independent experiments. ***$P < 0.001$ (Student's t-test referred to time 0). (B) HCT116 cells were treated with 25 μM PES or 250 nM STS and processed as in (A). (C) U87MG, HeLa, 293HEK and HL-60 were subjected for 12 h to 20, 25, 50 or 15 μM PES respectively. In parallel, cells were exposed to vehicle (V) or STS 250 nM for 12 h. Caspase activation (DEVDase activity) was quantified as above. (D) MEF defective in BAX and BAK proteins and their WT controls were treated for 24 h with 30 μM PES or, alternatively, with 1 μM STS. Cell viability was measured by the MTS reduction assay. Bar value is the mean ± S.D. of at least 3 independent determinations in 3 independent experiments.

when cells were treated with PES (Fig. 2D). Altogether these results indicated that PES was triggering caspase- and BAX/BAK-independent cell death in HCT116 and SH-SY5Y cells and suggested the involvement of a necrotic type of cellular demise.

3.2. PES induces a non-necroptotic, necrotic type of cell death

Depending on the cellular model, PES either elicits caspase-independent [2,19] or caspase-dependent [20–22] cell death, or a mixture of both [23]. To gain insight on the kind of cell death induced by PES, we evaluated the protective effects of commonly used inhibitors of death. Blockage of either apoptosis with the broad caspase inhibitor Q-VD-OPh, necroptosis with RIPK1 inhibitor necrostatin-1 or autophagy-driven cell death with 3-MA or Spautin-1, proved to be inefficient strategies to avoid PES-triggered cell death (Fig. 3A). Morphological studies by electronic microscopy remain one of the best methods to ascertain necrosis [9]. HCT116 cells displayed a morphological phenotype consistent with its origin in colon epithelia, with cells establishing junctions at the contact sites and displaying mitochondria and Golgi apparatus in a polarized way at a side of the nucleus (Fig. 3B). Upon PES treatment, HCT116 cells underwent mitochondrial disruption and an increased cytoplasm vacuolization (Fig. 3C). Later in the process, cell membrane became ruptured and the cytoplasm severely unstructured (Fig. 3D). These traits were consistent with a necrotic type of cell death. Similar morphological changes were observed in PES-challenged SH-SY5Y cells, with images of severely disrupted mitochondria only after 12 h of treatment (Fig. 3F). Together, these results indicated PES triggered a non-necroptotic, necrotic type of cell death.

3.3. ROS are pivotal elements in necrosis driven by PES

When the intracellular antioxidant systems are overloaded, the excess of ROS leads to oxidative stress, to the damage of essential biological components and, eventually, to necrosis [9]. In an attempt to establish a potential link between PES-driven necrosis and oxidative stress, we monitored the induction of ROS by PES in a time-dependent manner. At merely 30 min of PES treatment, we detected a significant increase in ROS content (Fig. 4A). To evaluate the relevance of the observed ROS in PES-driven necrosis, we assessed the effects of thiolic antioxidants, such as dithiothreitol (DTT) and N-acetyl cysteine (NAC) and confirmed they conferred a significant protection to PES-mediated necrosis in both SH-SY5Y and HCT116 cells (Fig. 4B and C). Efficient suppression of ROS by either DTT or NAC in combination with PES was controlled and confirmed by ROS quantification (data not shown). These findings were consistent with necrosis being a consequence of ROS build-up in response to PES. To further validate the central role of ROS in PES-induced necrosis, HCT116 and SH-SY5Y cells were challenged with combinations of PES and L-buthionine sulphoximine (BSO). BSO is a specific inhibitor of γ-glutamylcysteine synthetase able to deplete the intracellular levels of glutathione [24,25]. BSO and the subsequent glutathione depletion are not toxic for most cells unless subjected to oxidative stress [26]. Therefore, HCT116 and SH-SY5Y cells were pre-incubated with BSO and exposed to sublethal concentrations of PES. Under these circumstances, we reported a significant increase of ROS content (Fig. 4D). Thus supporting BSO improves generation of ROS by PES. Next, we applied this combination to our experimental paradigms and assessed the impact on their survival. Accordingly, BSO strongly cooperated with sublethal concentrations of PES to trigger

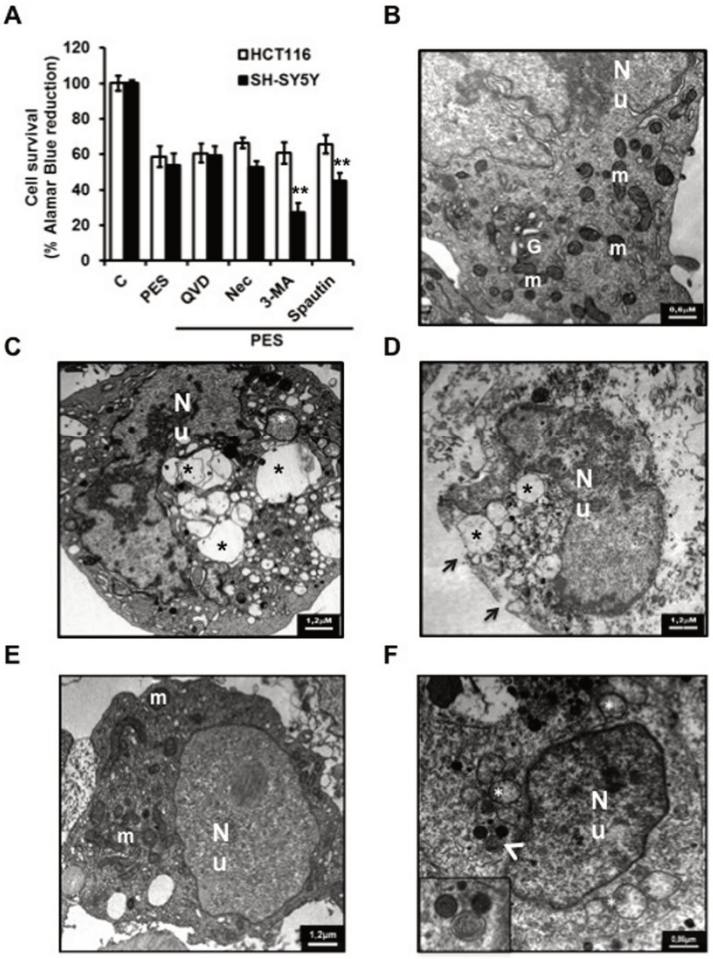

Fig. 3. PES triggers a non-necroptotic, necrotic type of cell death. (A) After a preincubation of 3 h with 10 μM QVD-OPh (QVD), 25 μM necrostatin-1 (Nec), 10 mM 3-MA (3-MA) or 10 μM Spautin-1 (Spautin), PES was added for 12 h at a concentration of 25 μM for HCT116 and 12.5 μM for SH-SY5Y cells. A control of vehicle-treated cells was included (C). Cell survival was quantified by Alamar blue. When a combined treatment was performed (PES + inhibitor), Alamar blue values were referred to the ones of the cells treated with the inhibitor alone. Bar value is the mean ± S.D. of at least 3 independent experiments with three independent measurements per experiment. $^{**}P < 0.005$ (Student's *t*-test referred to PES-treated control). (B) Transmission electron microscopy of HCT116 in untreated conditions. (C) HCT116 cells treated for 48 h with 25 μM PES, displaying an early necrotic phenotype. (D) HCT116 treated as in (C), showing a more advanced necrotic phenotype. Arrows indicate areas of discontinued plasmatic membrane. (E) Control SH-SY5Y cells untreated. (F) SH-SY5Y cells treated with 12.5 μM PES for 12 h. Arrowhead indicates the multilamellar autophagosome structure shown at higher resolution in the inset. Nu, nuclear chromatin; G, Golgi apparatus; m, mitochondria; black asterisks, vacuoles; white asterisks, dilated mitochondria with disrupted cristae.

cell death in HCT116 and SH-SY5Y cells (Fig. 4E). This cooperation was also detected in other cell lines, for instance U87MG and HeLa cells, which were more resistant to PES-deadly actions (Fig. 1A). Together, these findings reinforced the involvement of ROS in PES-triggered necrosis.

3.4. p53 participates in PES-driven necrosis

p53 is a pivotal stress sensor that responds to a great variety of cell insults by orchestrating cell demise. Besides apoptosis and autophagic cell death, p53 is also involved in necrosis [27–29]. To ascertain the involvement of p53 in PES-triggered necrosis, we used HCT116 cells and their p53-deficient counterparts. HCT116 p53−/− cells required higher concentrations of PES to obtain the same amount of cell death found in HCT116 cells, after 24 h of treatment (Fig. 5A). Indeed, leakage of the membrane in HCT116 p53−/− cells was significantly protected (15.6 ± 8.1% of PI-positive cells) when compared to HCT116 (45.5 ± 7.7% of PI-positive cells) (Fig. 5B). To better demonstrate the observed differences were due to p53 and not to unrelated phenomena, we reintroduced a functional p53 into HCT116 p53−/− cells. Transient transfection of p53 sensitized HCT116 p53−/− cells to PES-driven cell death (Fig. 5C). These data were in agreement with p53 being implicated in PES-driven necrosis.

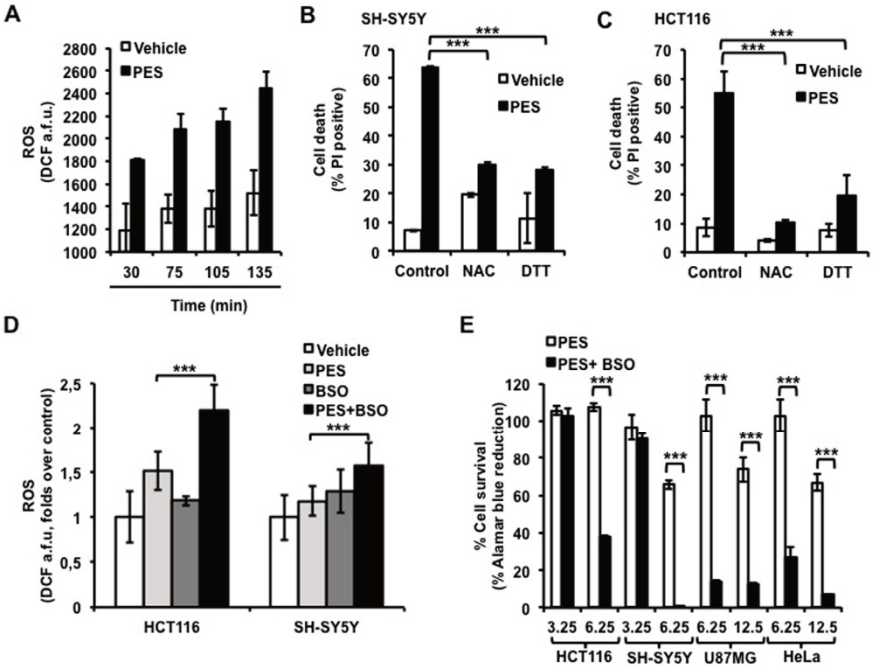

Fig. 4. ROS are pivotal elements in necrosis by PES. (A) HCT116 cells were treated with 25 μM PES or DMSO (Vehicle) for the times indicated in the *x*-axis. Quantification of ROS was performed as described in Section 2 and expressed as arbitrary fluorescence units (a.f.u) generated by DCF. (B) SH-SY5Y cells were pre-incubated for 1 h with 1.25 mM NAC or 500 μM DTT before adding 12.5 μM PES or DMSO (vehicle) for an additional 24 h period. Cell death was measured by flow cytometry and the count of PI stained cells. (C) HCT116 cells were treated with 25 μM PES for 48 h and analyzed as reported for SH-SY5Y in (B). ***$P < 0.001$ (Student's *t*-test referred to PES-treated control). (D) HCT116 and SH-SY5Y cells were either pre-incubated with BSO or left untreated. After a 3 h pre-incubation, PES was added at 6.25 μM for an extra 8 h period. ROS fluorescent signal was referred to the one of the DMSO (vehicle) treated cells. ***$P < 0.001$ (Student's *t*-test referred to PES-treated control). (E) Cell lines indicated in the *x*-axis were pre-incubated for 3 h with 50 μM BSO or left untreated. PES was then added at the concentrations reported in the *x*-axis. Cell survival was determined by the Alamar blue procedure. Bar value is the average ± S.D. of at least 3 independent experiments with 3 independent measurements. ***$P < 0.001$ (Student's *t*-test referred to PES-treated control).

3.5. p53 is predominantly located at the chromatin-enriched cellular fractions in response to PES

Since PES blocks the transcription-independent p53 translocation to mitochondria [1], we surmised the observed p53-dependent susceptibility could rely on its transcriptional activity, which consists in the regulation of a specific set of genes by binding to p53-responsive elements [30]. To do so, p53 needs to be located at the nucleus, more precisely at the chromatin-enriched fraction. Therefore, direct proof of this localization was necessary to substantiate p53 was modulating transcription of target genes. To experimentally find this evidence, we first interrogated the presence of p53 in chromatin-enriched fractions after challenging cells with PES. Using a previously described method to precisely separate cellular extracts into specific fractions [18], SH-SY5Y extracts were subfractionated into a cytoplasmic fraction, enriched in 0.1% triton-soluble proteins (C) and two nuclear fractions, one enriched in hydrosoluble proteins or nucleoplasm (N1) and the other, containing the insoluble and chromatin-bound proteins (N2). In healthy non-treated SH-SY5Y cells, most of the p53 content was found in either nucleoplasmic- or chromatin-enriched fractions. However, after PES treatment, p53 was preferentially found in the chromatin-enriched fraction (Fig. 6A). Precisely, p53 surpassed the control by 3 folds in the nucleoplasmic fraction and 6 folds in the chromatinic one (Fig. 6A). Purity of the fractions was controlled by the presence of the cytoplasmic enzyme lactate dehydrogenase (LDH) and nuclear polypyrimidine tract binding protein 1 (PTBP1). These data supported that PES was up-regulating p53, which located at the nucleus, where probably it was triggering the transcription of p53-responsive genes involved in a form of oxidative stress-regulated necrotic cell death. To validate this up-regulation in a time-dependent manner, total levels of p53 in SH-SY5Y cells were studied over a 24 h period of treatment. A notable increase of p53 content was evident at 6 h, before any sign of necrosis occurred (aprox. 2.4 folds over time 0, Fig. 6B). In parallel, abundance of one of the best-known transcriptional targets of p53, p21 [31,32], was monitored. Kinetics of p21 paralleled those of p53 (Fig. 6B). Similar results were obtained with HCT116 cells (not shown). Overall, these results were suggestive of a functional p53, activating transcription in response to PES. Finally, we considered that specific inhibition of p53 would be of paramount importance to assess its role in cell death. To do so, endogenous p53 was specifically silenced with a lentivirus carrying an shRNA against p53 (shp53). We observed an increase in the resistance of HCT116 (Fig. 6C) and SH-SY5Y (Fig. 6D) cells to PES-induced necrosis in any of the concentrations employed. Efficiency of p53 down-regulation was assessed and found to be greater than 80% by Western-blot (data not shown). Therefore, we concluded that a p53-dependent program was involved in necrosis triggered by PES.

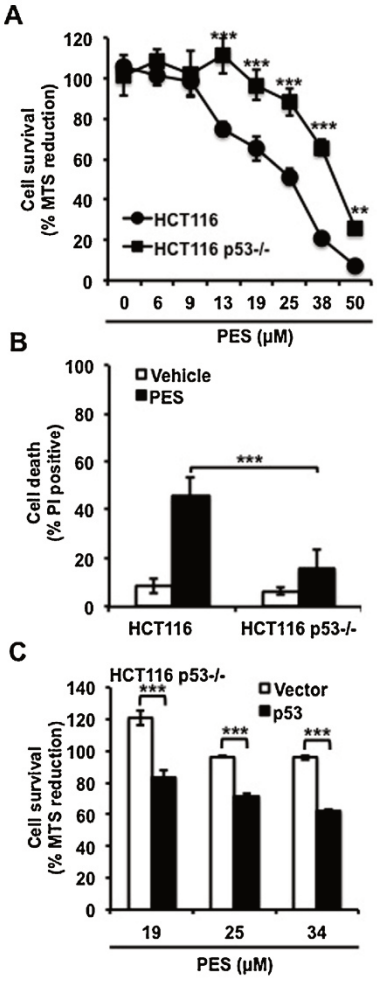

Fig. 5. p53 participates in PES-driven necrosis. (A) HCT116 WT and HCT116 p53−/− cells were challenged with the indicated concentrations of PES for a period of 24 h. Cell survival was assessed by MTS reduction. Plots represent the average ± S.D. of at least 3 independent experiments with 3 independent determinations. (B) HCT116 and HCT116 p53−/− were treated for 48 h with 25 μM PES. Cell death was determined by flow cytometry count of PI stained cells. Bar value equals mean ± S.D. of at least 3 independent experiments with 3 independent measurements. (C) HCT116 p53−/− cells transfected with a plasmid carrying a functional p53 (p53) or empty (vector) were subjected to PES treatment. Cell survival by MTS reduction was evaluated after 24 h. Results are the average ± S.D. of at least 3 independent experiments with 3 independent determinations. $^{**}P < 0.005$; $^{***}P < 0.001$ (Student's *t*-test).

3.6. ROS and p53 form a positive feedback loop in PES-mediated necrosis

p53 can either be activated in response to oxidative stress or, alternatively, generate ROS through transcription-dependent mechanisms [7]. Several experiments were performed to distinguish between these two possibilities. First, SH-SY5Y cells were either treated with PES plus or minus DTT and p53 content was assessed by Western-blot (Fig. 7A). Cells challenged with PES up-regulated p53 by 35 folds compared with untreated cells. Although single DTT treatment increased p53 by 10 folds, combination of DTT and PES resulted in a 15 folds increase. In other words, in the presence of DTT, PES triggered a mere increase of 1.5 folds over DTT, indicating that p53 up-regulation was mainly a response to the generation of ROS. Second, we evaluated the production of ROS in p53-null cells stimulated with PES. At times when p53 was up-regulated by PES, HCT116 p53−/− cells generated significantly lower amounts of ROS than HCT116 cells (Fig. 7B). In sum, we showed ROS up-regulated p53 and p53 promoted ROS generation. Therefore we propose the existence of a positive feed back loop between p53 and ROS in the process of PES-mediated necrosis.

4. Discussion

Here, we describe PES induces cytotoxicity in cell lines of different ontogeny. Cell death by this compound is caspase-independent and presents a prominent cytoplasmic vacuolization. The nuclei of PES-injured cells do not show morphological traits of apoptotic cell death. Moreover, PES promotes an early induction on the intracellular ROS content that, in turn, is a key event for the cytotoxic process. This oxidative stress is responsible of an up-regulation of p53, which participates in PES-driven cell death. Notably, down-regulation or genetic suppression of p53 makes cells more resistant to necrosis induced by PES. Localization of p53 in the chromatin-bound fraction and up-regulation of p21 indicate the involvement of a transcription-dependent p53 program in PES-driven necrosis. Finally, we also present evidence of PES eliciting a positive feedback loop between ROS and p53, which results in the final necrotic outcome.

p53, also known as the "guardian of the genome", is a pivotal element in the cell response to chemotherapeutic agents. The fact that p53-defective cells remain sensitive to PES-induced cell death is taken as a proof of its irrelevance in the process [2,21]. Nonetheless, up-regulation of p53 in response to PES is reported in multiple cell lines [2,3]. There exists only one exception in which PES triggers p53-independent apoptosis, without p53 up-regulation [21]. Under these premises, we interrogated the involvement of p53 in genetically modified experimental paradigms: HCT116 and their isogenic HCT116 p53−/−, HCT116 p53−/− cells over-expressing a functional p53, and, finally, HCT116 and SH-SY5Y cells with diminished levels of p53. Regardless of the genetically modified paradigm, p53 exerted a positive role, increasing PES-triggered necrosis. Moreover, we have provided evidences of p53 transcriptional involvement since p53 remains in the appropriate nuclear compartment for transcription modulation and content of p21 is increased.

Blockade of RNA polymerase II-mediated transcription induces p53 accumulation in mitochondria, being the critical factor for eliciting p53-dependent, but transcription-independent, apoptosis [33]. On the contrary, PES is a well-known inhibitor of p53 translocation to mitochondria [1] with no reported effects on transcription. Applying the former reasoning, PES-driven global increase of p53, having its movement to the mitochondria impeded, could act as a permissive event for p53 transcription-dependent necrosis to occur. Despite our findings, high concentrations of PES are cytotoxic in HCT116 p53−/− cells. Our explanation to this phenomenon is the loss of drug specificity at the highest concentrations employed. Our data also evidence that PES triggers cell death in p53-null cells, such as HL-60. In this regard, when high concentrations of PES are used, we cannot rule out the involvement of other members of the p53 family of transcription factors, such as p63 or p73, which all share amino acid sequence identity in the DNA-binding domain, and thus could present redundant functions in the regulation of gene expression [34].

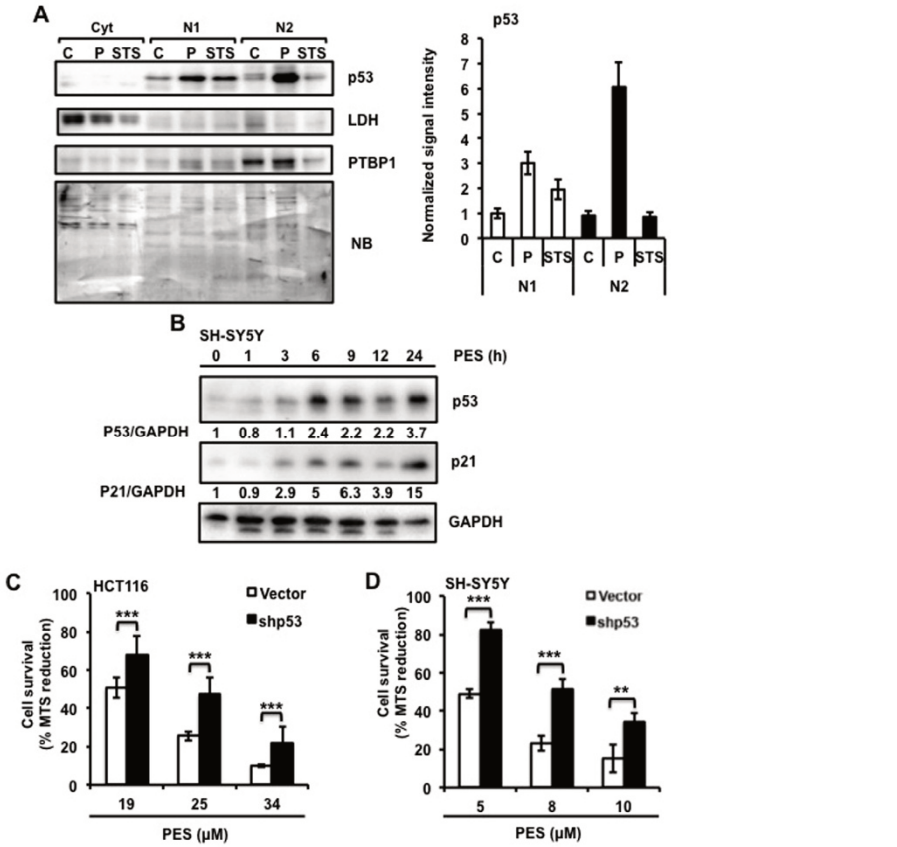

Fig. 6. p53 is predominantly located at the chromatin-bound subfractions in response to PES. (A) SH-SY5Y cells were treated for 6 h with PES 12.5 μM (P), Staurosporine 1 μM (STS) or left untreated (C). Cells were next subjected to subfractionated extractions as reported in the text. Cytosolic (Cyt), nucleoplasmic (N1) and chromatin-enriched (N2) extracts were obtained. These extracts were analyzed by Western-blot with p53, LDH and PTBP1 antibodies as stated in the figure. NB refers to the membrane stained with naphtol blue to assess an even loading of protein per fraction. Next to the image the quantitation of p53 content in N1 and N2 fractions expressed as "normalized signal intensity" to the p53 in left untreated extracts (C) and performed as described in Section 2. Results are the average ± S.D. of 2 independent experiments. (B) SH-SY5Y cells were treated with PES 12.5 μM for the indicated times. Total extracts were obtained and p53 and p21 content was detected by Western-blot. GAPDH was used to control protein loading. The quantification of p53 and p21 content referred to GAPDH is indicated below each panel. Image is the result of one representative experiment out of two. (C) HCT116 cells were infected with a virus carrying a shRNA against p53 or an empty one. Cell survival was assessed by MTS and referred to the values of vehicle treated cells. Results are the average ± S.D. of at least 3 independent experiments with 3 independent measurements per experiment. (D) SH-SY5Y cells were processed and viability assessed as stated before in C for HCT116. $^{**}P < 0.005$; $^{***}P < 0.001$ (Student's *t*-test).

The critical role of ROS in the regulation of necrotic pathways [35,36], prompted us to characterize them in our paradigms. Oxidative stress occurs as a consequence of the imbalance between the rate of ROS production and neutralization by specific detoxifying proteins, frequently leading cells to death either by apoptosis or necrosis. We found an accumulation of ROS at times as early as 30 min after PES treatment. Moreover, thiolic antioxidants conferred the highest protection facing PES when compared to the other compounds tested (Fig. 3A). To our knowledge, only one report exists about ROS involvement in the lethal mode of action of PES [22]. However, the authors show that PES triggers apoptosis rather than necrosis. Two main issues need to be taken into account in their work: the delayed kinetics of ROS accumulation, starting at 4 h of treatment, and the non-tumor origin of their cellular model, which is based on rainbow trout, gill-derived, cells. In addition we report a pharmacological cooperation between PES and BSO that translates into an increased ROS content and a greater cytotoxicity. This cooperation reinforces our conclusion about PES-mediated ROS generation but, in addition, it raises strategic implications for cancer therapy. In our laboratory, we have observed BSO to be minimally toxic for cells unless subjected to other simultaneous stresses. This is consistent with BSO being well tolerated by humans in clinical assays [37,38]. The association of BSO and anticancer drugs is not new, it has been described, for example, with retinoids in neuroblastoma [39], melphalan or cisplatin in ovarian cancer [40,41], vitamin D in breast cancer [42], or azathioprine in colon and hepatic carcinoma [43]. We have no knowledge about PES toxicity in humans. However, BSO combined to PES will probably allow the reduction of PES doses, not decreasing its efficacy and minimizing its nonspecific effects.

Multiple lines of evidence exist on the mutual regulation of p53 and ROS. For instance, DNA damage by ROS triggers an alarm response with p53 as the central player. Moreover, ROS activate phosphorylation cascades trough p38α MAPK and ERK ending up

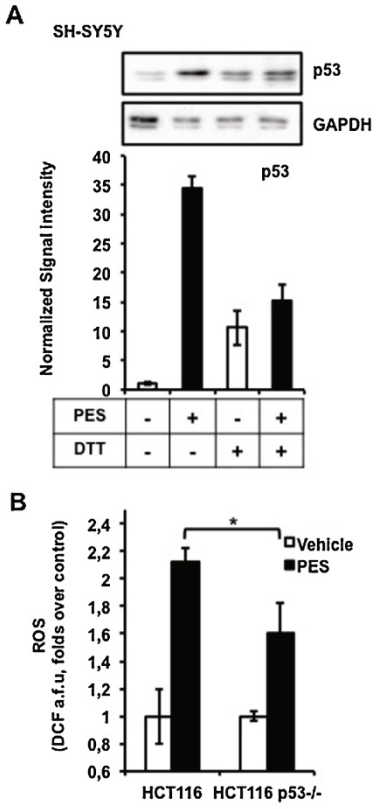

Fig. 7. ROS and p53 mutually regulates each other in response to PES. (A) SH-SY5Y cells were pre-incubated with 500 μM DTT or left untreated. Then exposed to 12.5 μM PES or vehicle for 6 h. Total protein extracts were performed and analyzed by Western-blot with p53 and GAPDH antibodies. GAPDH was used to control the protein load. Below, quantitation of the p53 expressed as "normalized signal intensity" to the p53 in left untreated extracts and performed as described in Section 2. Results are the average ± S.D. of 2 independent experiments. (B) HCT116 and HCT116 p53−/− isogenic cells were treated with 25 μM PES for 6 h. Quantification of ROS was referred to a control with DMSO (vehicle). Quantification procedure is described in Section 2 and expressed as arbitrary fluorescence units (a.f.u) generated by DCF. *$P < 0.01$; (Student's *t*-test).

in p53 stabilization [44]. Finally, oxidation of cysteine residues in p53 modifies its DNA binding activity and the transcription of specific genes [7]. Our data evidenced ROS inhibition attenuates p53 increase in response to PES, thus positioning ROS upstream of p53 up-regulation. On the other hand, p53 can promote ROS generation by transcriptionally inducing enzymes, such as quinone oxidoreductase or proline oxidase, or pro-oxidant genes, such as *BAX*, *PUMA* and $p66^{Shc}$ [7]. Similarly, p53-mediated suppression of antioxidant genes such as manganese superoxide dismutase (*MnSOD*), could finally impinge on the ROS content [7]. Though p53 is known to respond to ROS by inducing either anti-oxidant or pro-oxidant genes, it has been suggested that the severity of the stimulus could be critical in making this decision [45]. In support of the pro-oxidant role of p53, we found that HCT116 p53−/− cells generate a limited amount of ROS when compared to HCT116 (Fig. 7B).

Our results using multiple cell lines are consistent with previous data reporting PES elicits a caspase-independent cell death characterized by a prominent cytoplasmic vacuolization [2]. The same authors report a null effect of up-regulating the anti-apoptotic BCL-X_L protein on PES-induced cell demise. In the same line, we have found that MEF *Bax*−/−*Bak*−/− cells remain fully sensitive to PES-triggered cell death. Since these cells are fully resistant to apoptosis through the intrinsic pathway [15], these data prove that death pathways engaged by PES are clearly unrelated to apoptosis. Consistently, in primary effusion lymphoma PES triggers a caspase-independent cell death mediated by lysosomal permeabilization [19]. Nonetheless, in other cellular models, apoptosis seems to be the main type of cell death caused by PES [20–22]. To complete the picture, a simultaneous mixture of both caspase-dependent and independent cell death was found in pancreatic cell lines treated with PES [23]. These data, which seem to be contradictory, can be explained by differences in the cell death behavior, inherent to each cellular model. Alternatively, taking into account our results, another plausible explanation would be the different cellular ability to buffer PES-driven oxidative stress. Indeed, it is broadly accepted that ROS have the capability to modulate apoptosis. For instance, low concentrations of H_2O_2 trigger caspase-dependent apoptosis while higher amounts elicit necrosis and impair caspase activation [46]. In this sense, it is known that direct oxidation of the cysteine from the catalytic center of caspases is able to inhibit these proteases [46]. Along the same line, our results prove first, that PES is unable to promote the activation of caspases in multiple cell lines (Fig. 2A–C) and second, that the pan-caspase inhibitor Q-VD-OPh is unable to protect cells from PES-driven necrosis (Fig. 3A).

Pioneer studies cataloged cell death processes into three main morphological categories: apoptosis, autophagic cell death and necrosis. Through time, the need of a more precise definition has become evident and the evaluation of new biochemical parameters has implemented the original classification [47]. Similarly, pharmacological strategies have been set up to distinguish between these processes of cell death. 3-MA and Spautin-1 are compounds known for its activity suppressing autophagy [48–50]. Our data showed neither 3-MA nor Spautin-1 were able to block cell death in response to PES. The absence of protection suggests the induction of autophagy is not involved in the mechanism of death by PES. Additionally, it is known that PES impairs the autophagy–lysosome systems, resulting in a accumulation of autophagic vacuoles and generation of proteotoxic stress [2,19]. Furthermore, combination of PES with 3-MA or Spautin-1 enhances cytotoxicity in SH-SY5Y cells, thus suggesting autophagy could be playing a protective role, instead. MEFs *Atg5*−/− are unable to perform the classical autophagy pathway [51] and thus, they are a valuable tool to study the involvement of autophagy under different settings. Experiments from our laboratory have revealed that MEFs *Atg5*−/− display a greater susceptibility to PES-driven death (data not shown). We believe these results are consistent with the role of autophagy attenuating proteotoxicity [52] and thus, diminishing the proteotoxic stress inflicted by PES.

Necrosis is morphologically characterized by rounding of the cell, a gain in cell volume (also known as oncosis), organelle swelling, lack of internucleosomal DNA fragmentation, and plasma membrane rupture [53]. Despite its early association to accidental forms of cell death, new data link it to regulated forms of cell demise, otherwise known as "programmed necrosis". In this sense, necroptosis is currently the best characterized form of programmed necrosis [54]. In our experiments, the absence of protective effects of the combination of necrostatin-1 and PES, indicates PES triggers a non-necroptotic necrosis. While the physiological relevance of alternative forms of cell death remains under study, there is no doubt about the spontaneous appearance of mechanisms to elude them. These mechanisms are clearly linked to chemoresistance in cancer. For instance, overexpression of

PDIA6, a protein disulfide isomerase, is one of the mechanisms accountable for the increased chemoresistance of lung adenocarcinoma to cisplatin-induced necroptosis [55]. Similarly, ROS-induced miR-21 promotes apoptotic resistance of vascular smooth muscle cells to oxidative stress [56]. Infectious myocarditis presents both forms of cell death: apoptosis and necrosis. Forced expression of miR-21 reduces apoptotic myocarditis through the down-regulation of programmed cell death 4 (PDCD4) messenger [57]. Nevertheless, the overexpression of the same microRNA has no impact reducing necrotic-type myocarditis [57]. In conclusion, we believe that inducers of non-canonical forms of programmed necrosis harbor a great potential for new-line pharmacological therapies.

Acknowledgments

We thank Dr. Mario Encinas, Dr. Xavier Dolcet and Dr. Núria Eritja for providing the FSV and FSV shRNA p53 vectors. We are grateful to Dr. Gabriel Gil for supplying the pCMV-Neo-Bam and pCMV-Neo-Bam p53 plasmids.

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3 Cell death induced by 2-phenylethynesulfonamide uncovers a pro-survival function of BAX

Cancer Letters 354 (2014) 115–121

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Article

Cell death induced by 2-phenylethynesulfonamide uncovers a pro-survival function of BAX

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ARTICLE INFO

Article history:
Received 20 May 2014
Received in revised form 22 July 2014
Accepted 30 July 2014

Keywords:
Cell death
Mitochondrial dynamics
BAX
BAK
Pifithrin-μ
Mdivi-1

ABSTRACT

PES (2-phenylethynesulfonamide) was initially identified as an inhibitor of p53 translocation to mitochondria and named Pifithrin-μ. Further studies showed that PES selectively killed tumour cells and was thus a promising anticancer agent. PES-induced cell death was characterised by a non-apoptotic, autophagosome-rich phenotype. We observed this phenotype via electron microscopy in wild type (wt) and double *Bax-/- Bak-/-* (DKO) mouse embryonic fibroblasts (MEFs) treated with PES. We excluded the involvement of effector caspases, BAX and BAK, in causing PES-triggered cell death. Therefore, apoptosis was ruled out as the lethal mode of action of PES. Surprisingly, MEFs containing BAX were significantly protected from PES treatments. BAX overexpression in *Bax-/-* MEFs confirmed this pro-survival effect. Moreover, this protective effect required the ability of BAX to localise to mitochondrial membranes. Conversely, mitochondrial fusion induced by treatment with Mdivi-1 conferred increased resistance to MEFs subjected to PES treatment. The involvement of BAX in the regulation of mitochondrial dynamics has been reported. We propose the promotion of mitochondrial fusion by BAX to be the pro-survival function attributed to BAX.

Introduction

Pifithrins are small synthetic molecules that can inhibit the apoptosis-inducing functions of the p53 protein. Pifithrins were created to reduce the toxicity associated with radiation and chemotherapy in the context of cancer treatment, which is largely mediated by p53. Pifithrin-α was the first compound identified and characterised as an inhibitor of the transcriptional activity of p53 [1]. However, p53 can cause apoptotic cell death via mechanisms that are independent of transcription. For instance, p53 can translocate to mitochondria, interact with the anti-apoptotic members of the Bcl-2 family of proteins, neutralise them and cause apoptosis [2]. This fact prompted new analyses of chemical libraries and the discovery of pifithrin-μ. Pifithrin-μ can disrupt the interactions between the p53 and Bcl-2 proteins without modifying the transcriptional activity of p53. Consistently, it conferred a partial protection to mice subjected to γ-radiation [3].

Further studies demonstrated that pifithrin-μ was an effective inducer of cell death and that cancer cells are more sensitive to this drug [4,5]. The characterisation of this death process showed it to be independent of p53 and caspases. The cell morphology was characterised by increased vacuolisation and abundant autophagic vesicles. However, this change was not due to the promotion of autophagy but rather to the blockage of the autophagic flux. Therefore, proteotoxic stress, defined as the accumulation of misfolded proteins, was proposed as the mechanism of pifithrin-μ-induced cell death [4,6]. Moreover, in addition to p53, pifithrin-μ targeted the heat-shock protein 70 (HSP70) family of proteins [4,7]. The subsequent disruption of its chaperone function was very consistent with the resulting proteotoxicity. The identification of targets different from p53 prompted the renaming of pifithrin-μ to PES (the acronym of 2-phenylethynesulfonamide) [4]. We adhere to this naming convention hereafter.

Although HSP70 and proteotoxicity explained the lethal properties of PES quite satisfactorily, controversy arose about the type of cell death this drug induced. Some researchers were clearly finding a caspase-independent, non-apoptotic, type of cell death [4,7]. On the contrary, other researchers were finding canonical apoptotic processes [5,8,9]. Moreover, in pancreatic cell lines, a mixture of caspase-dependent and independent processes were identified [10]. Most models of apoptosis depend on mitochondrial outer membrane permeabilisation (MOMP), an event mediated by the BAX and BAK proteins. These proteins define the intrinsic or mitochondrial

Abbreviations: DKO, double knock out; GFP, green fluorescent protein; MEFs, mouse embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilisation; PES, 2-phenylethynesulfonamide; SEM, standard error of mean; STS, staurosporine; wt, wild type.

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http://dx.doi.org/10.1016/j.canlet.2014.07.037
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pathway of apoptosis. Cells deficient in both proteins become totally resistant to apoptosis induction via the intrinsic pathway [11,12]. Double *Bax-/- Bak-/-* (DKO) mouse embryonic fibroblasts (MEFs) and their wild type (wt) counterparts provide the most conclusive system for assessing the implication of the mitochondrial mechanisms that trigger apoptosis. Our aim was to explore the lethal effects of PES on this system. Unexpectedly, we found that more wt MEFs survived than DKO MEFs after a treatment with PES. PES did not activate either the effector caspases or the intrinsic apoptotic pathway. Therefore, apoptosis could be excluded as the type of cell death triggered by PES. The protein BAX was identified as promoting the increase in cell survival in wt MEFs. Upon induction of mitochondrial fusion, we found that MEFs became more resistant to PES-induced cell death. We hypothesised that the pro-survival action of BAX was based on the modulation of mitochondrial dynamics.

Materials and methods

Cell lines and drug treatments

Immortalised embryonic fibroblasts derived from mice (MEFs) knocked out for *Bax* (*Bax-/-*), *Bak* (*Bak-/-*), both genes (DKO) and their wild type (wt) counterparts were obtained from the laboratory of the late Prof. Korsmeyer [11,12]. These MEFs have been an important tool in our previous pharmacological studies [13,14]. The HeLa cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell types were grown in 2 mM L-glutamine-supplemented DMEM medium that contained 10% of foetal calf serum (FCS). The media and FCS were provided either by Invitrogen or Lonza (Barcelona, Spain). Plasmocin™ (5 μg/ml) was used as the antibiotic in the medium (InvivoGen, San Diego, CA, USA). The general culturing conditions were 37 °C and a water-saturated, 5% CO_2 atmosphere. The culture dishes and other plastic disposable tools were supplied by Bibby Sterilin (Staffordshire, UK) and Becton Dickinson (Franklin Lakes, NJ, USA). PES (2-phenylethynesulfonamide), i.e., pifithrin-μ, staurosporine (STS) and Mdivi-1 were purchased from Sigma (St. Louis, MO, USA). The stock solutions were prepared in DMSO. The drugs were serially diluted from these stock solutions or delivered to the media. The final concentrations are reported in the text and figures.

Cell death and caspase activity assessment

To quantify the ratios of cell death in the culture plates, the Cell Titer 96® and Cytotox 96® kits were used. Cell Titer 96® measured the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS). Cytotox 96® measured the amount of lactate dehydrogenase (LDH) released from dying cells. Both kits were supplied by Promega Biotech Ibérica, SL (Barcelona, Spain). Because MTS reduction alone can lead to misleading interpretations due to a drug-induced cessation in cell proliferation, we supplemented the MTS data with LDH measurements. The combination of both procedures allows a robust determination of cell death, notwithstanding the type of death [13–15]. Apoptosis is a phenotype of cell death that depends on effector caspase activation. This activation can be quantified based on the cleavage of a synthetic substrate (Ac-DEVD-afc) and the subsequent fluorescence released (DEVDase activity), as we have reported previously [16].

Protein extraction and western blotting

The cells were lysed in a buffer containing 100 mM Tris/ClH pH 6.8, 1% SDS, 1 μM EDTA, plus a cocktail of protease inhibitors from Sigma and subjected to sonication. The protein extract was obtained after a centrifugation at 12 000 g for 15 minutes. The protein concentration was determined, and an equal amount was loaded per lane for SDS 12%-polyacrylamide gel electrophoresis. Following electrotransfer to 0.45 μm PVDF membranes (Immobilon™) from Merck Millipore (Barcelona, Spain), immune detection was performed with the following primary antibodies: anti-BAX rabbit polyclonal IgG (Upstate catalog 06-499) and anti-BAK rabbit polyclonal IgG (Upstate Catalog 06-536), which were both diluted to 2 μg/ml and supplied by Merck Millipore (Barcelona, Spain). Glyceraldehyde-3-P-dehydrogenase (GAPDH) was detected with the peroxidase-conjugated monoclonal antibody from Sigma. This monoclonal antibody was employed at a dilution of 1:4000 to assess the amount of protein loaded per lane. The Immobilon™ reagent from Merck Millipore was employed as the chemiluminescent peroxidase substrate.

Transient transfections

DKO MEFs were plated in p35 wells at 60–80% confluence. After 24 hours, 4 μg of plasmid DNA was transfected using the TurboFect reagent (Fisher Scientific, Barcelona, Spain) according to the manufacturer's instructions. The plasmids employed were hBax C3-EGFP (Addgene #19741), hBax S184E C3-EGFP (Addgene #19743), EGFP-Bak (Addgene #32564) and the empty vector (pEGFP-C3). These plasmids were originally used to explore the functional domains of the BAX protein [17]. The cells were then allowed to recover for an additional 24 hours, harvested, plated at 6000 cells/well in M96 plates and subjected to drug treatment.

Microscopic morphological studies

For electron microscopy, cells from treated or untreated cultures were collected, washed twice in PBS (150 mM ClNa, 2.7 mM ClK, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and fixed for 30 minutes at 4 °C in 100 mM phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde. After rinsing the pellets twice with PBS at 4 °C, the cells were post-fixed in buffered OsO_4, dehydrated in graded acetone and embedded in Durcupan® ACM resin (Fluka, Buchs, Switzerland). Ultrathin sections mounted on copper grids were counterstained with uranyl acetate and lead citrate. A transmission electron microscope (Zeiss EM 910) was employed to visualise the cellular ultrastructure.

For the fluorescence microscopy of mitochondria, plated cells were placed in PBS containing MitoTracker® Red (Life Technologies, Barcelona, Spain) at a concentration 50 nM for 30 minutes. After rinsing, the plated cells were maintained in complete medium and observed with an inverted fluorescence microscope.

Results

Cell death induction by PES was caspase-independent and more prominent in the DKO MEFs

To circumscribe the role of BAX and BAK in the cell death process triggered by PES, we characterised its lethal effects in DKO MEFs and their wt counterparts. Both cell lines were treated with increasing concentrations of PES, and the cell viability was determined after 8 hours of treatment. The cell viability was measured using the MTS procedure and found to decrease in a concentration-dependent manner (Fig. 1A). To confirm this result, both cell lines were subjected to identical PES concentrations, and the cell death was measured with the LDH procedure after 24 hours of treatment (Fig. 1B). The cell death and viability values were complementary and consistently proved the greater resistance of wt MEFs. In parallel, these cell lines were treated with staurosporine (STS) at a concentration of 1 μM for 8 hours (MTS assay) and 24 hours (LDH assay). STS is one of the most widely accepted inducers of the intrinsic or mitochondrial pathway of apoptosis, i.e., the pathway mediated by BAX and BAK. As expected, DKO cells but not the wt MEFs were resistant.

Because apoptosis can also be triggered in a BAX- and BAK-independent manner via the extrinsic pathway, we studied the activation of effector caspases in wt MEFs treated with PES (Fig. 1C). The cells were treated for 12 hours with increasing concentrations of PES and STS (200 nM) to control apoptosis and caspase activation. STS was observed to increase the caspase activity more than 10-fold compared to the basal value of the untreated cells. Conversely, the PES-induced values did not surpass the basal level, irrespective of the concentration employed. Moreover, they fell below the basal level in a concentration-dependent manner. In conclusion, the lack of effector caspase activation and non-involvement of BAX and BAK rule out apoptosis as the lethal mode of action of PES. In addition, PES highlighted a pro-survival effect for BAX, BAK, or the combination of these proteins.

BAX protein was protecting MEFs from PES-induced cell death

We performed a new series of experiments in order to determine the involvement of BAX, BAK or the combination of these proteins in protecting cells from PES. The effects of PES on *Bax-/-*, *Bak-/-*, DKO and wt MEFs were tested in parallel (Fig. 2A). *Bax-/-* and DKO MEFs were similarly sensitive and displayed the greatest susceptibility to PES lethality. Conversely, *Bak-/-* and wt MEFs wt were clearly more resistant to PES. To better quantify the sensitivity of these cells lines to PES, we calculated the drug concentration required to kill 50% of the cell population (LC_{50}). Based on our data

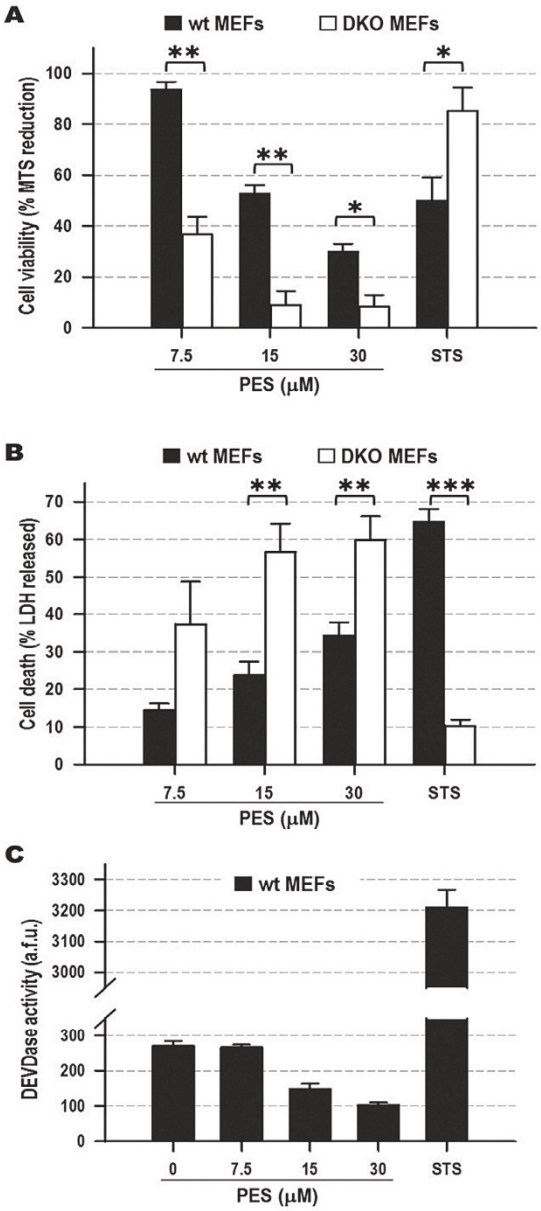

Fig. 1. MEFs defective in both, BAX and BAK proteins, displayed increased sensitivity to PES toxicity. As stated, wt and DKO MEFs were treated with increasing concentrations of PES and STS (1 μM). Cell viability was determined using the MTS procedure after 8 hours of treatment. The bar value is the mean ± SEM, n = 4, each n = 5–6 replicates (A). Cell death was determined by the LDH procedure after 24 hours of treatment. The bar value is the mean ± SEM, n = 4, each n = 3 replicates (B). PES did not activate effector caspases in wt MEFs. As stated, wt MEFs were treated with increasing concentrations of PES and STS (200 nM). After 12 hours of treatment, the cells were lysed and the DEVDase activity quantified in arbitrary fluorescent units (a.f.u.) The bar value is the mean ± SEM of 6 replicates (C). Student's t-test: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

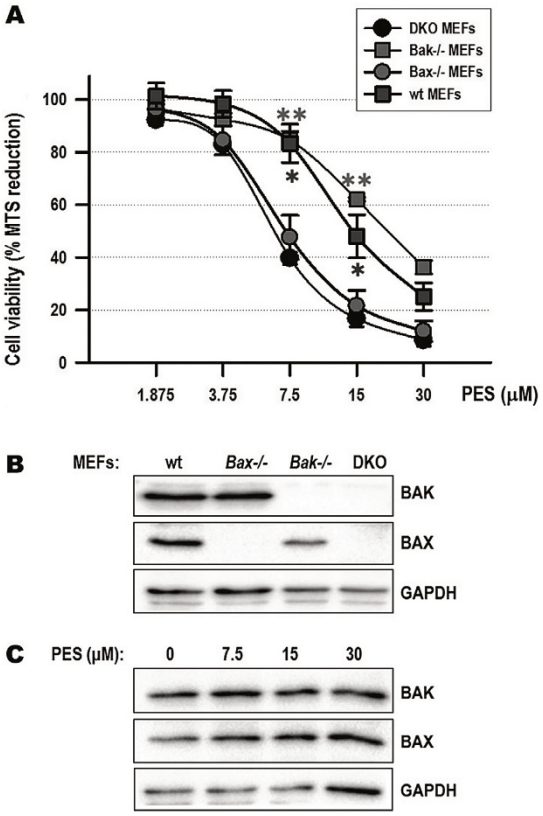

Fig. 2. MEFs deficient in BAX protein displayed increased sensitivity to PES. DKO, *Bax*-/-, *Bak*-/- and wt MEFs were subjected to increasing concentrations of PES as stated in the graph. Cell viability was measured using the MTS procedure after 8 hours of treatment. The values are the mean ± SEM, n = 6, each n = 5–6 replicates. Student's t-test is referred to DKO values: $^{*}P < 0.05$, $^{**}P < 0.01$ (A). BAX and BAK content assessment. Protein extracts were obtained from DKO, *Bax*-/-, *Bak*-/- and wt MEFs and analysed using western blots with antibodies recognising BAX and BAK protein, as indicated (B). PES treatment did not affect the BAX and BAK contents. As stated, wt MEFs were treated for 8 hours with increasing concentrations of PES, and the proteins were extracted and analysed by western blotting as stated above (C) The amount of GAPDH was detected via immunoblotting to assess the amount of protein loaded per lane.

(Fig. 2A), the LC_{50} was 6.5 μM for DKO, 7.25 μM for *Bax*-/-, 14.5 μM for wt and 21.5 μM for *Bak*-/- MEFs. Therefore, wt MEFs doubled and *Bak*-/- MEFs approximately tripled the values of their *Bax*-/- and DKO counterparts. In conclusion, the presence of BAX seemed to be the crucial element correlating with resistance to PES-induced cell death.

The content of BAX and BAK proteins was routinely assessed in the four cells lines using western blotting and found to be consistent with expected values (Fig. 2B). This determination excluded mistakes due to mislabelling or the accidental cross-contamination of the cell lines. However, PES could change the cell content of BAX and BAK proteins by modulating their expression or degradation rates. The wt MEFs were treated with increasing concentrations of PES for 8 hours, and the content of BAX and BAK proteins was assessed with a western blot (Fig. 2C). As shown, PES did not significantly affect the amounts of BAX and BAK proteins. Because epigenetics or other uncontrolled phenomena could influence the

response to PES in each type of MEF, we adopted another experimental approach. We transiently transfected DKO MEFs with plasmids to overexpress the BAX, BAXS184E and BAK proteins. BAXS184E contains a Serine 184 to glutamic mutation in the C terminus transmembrane domain. This mutation is known to impair the location of BAX to mitochondrial membranes [17]. These proteins are fused with the green fluorescent protein (GFP), and therefore, the efficiency of the transient transfection can be assessed by fluorescence microscopy. Experiments were only continued if the efficiency rated 50% of the cell population. As shown, the non-mutated *Bax* gene conferred a statistically significant protection to DKO MEFs (Fig. 3A). Neither the *Bax*S184E nor *Bak* gene promoted any survival effect after being transfected. The effect of STS, a canonical inducer of apoptotic cell death, was tested in parallel to assess the function of the transfected proteins. The transfection of the *Bax* and *Bak* genes increased cell death in accordance with their apoptosis mediating role. The transfection of the *Bax*S184E mutant was significantly less lethal than that of the *Bax* gene, which agreed with its impaired functionality (Fig. 3B). In conclusion, the BAX pro-

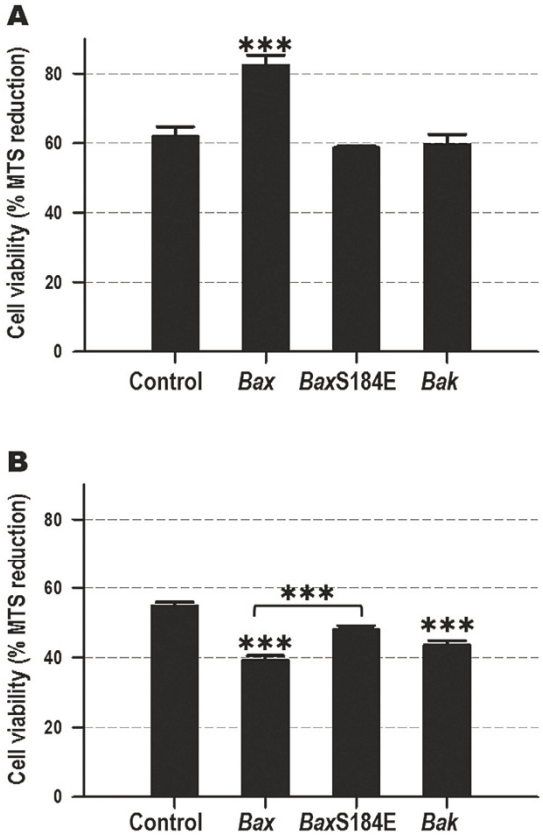

Fig. 3. BAX protein conferred resistance to PES-induced lethality to the cells. DKO MEFs were transiently transfected with the genes indicated in the x-axis. The efficiency of transfection approached 50% of the cells in culture. After a treatment with PES (7.5 μM) for 24 hours, the cell viability was assessed using the MTS procedure (A). As a functional control, the cells were treated in parallel with STS (100 nM) for 24 hours and subjected to the MTS test (B). The bar value is the mean ± SEM, n = 1, n = 5–6 replicates, representative of 3 independent experiments. Unless otherwise indicated, Student's t-test is referred to control: ***$P < 0.001$.

survival effect in cells treated with PES was proven and shown to be dependent on its appropriate insertion into the mitochondrial membranes.

Mitochondrial fusion was observed in wt MEFs treated with PES

In our initial studies of PES-induced lethality, wt and DKO MEFs were treated with 30 μM PES for 7 hours and subjected to electron microscopy analysis. Healthy, untreated wt MEFs displayed a typical fibroblast morphology with a developed reticulum and abundant mitochondria in the cytoplasm. The nuclei were quite large and contained discernible heterochromatin condensations located preferentially adjacent to the nuclear membrane (Fig. 4A). Upon PES treatment, a prominent vacuolisation was observed in the cytoplasm (Fig. 4B). The nuclei lost their peripheral heterochromatin but remained well preserved. No apoptotic features were detected. When the resolution of the images was increased, abundant multilamellar autophagosome structures that characterise autophagy were observed (Fig. 4C, arrows). This cell death phenotype reinforced the occurrence of a non-apoptotic type of cell death and reproduced the type previously reported in other cells [4]. DKO MEFs subjected to PES treatment were also studied and displayed a death pattern similar to the one described for the wt MEFs (not shown). However, in PES-treated cells, branched and fused mitochondrial assemblies displaying cristae with a normal morphology were only found in wt MEFs (Fig. 4D, arrowheads). This finding seemed to indicate that mitochondrial fusion was more common if the BAX protein was present.

Increased mitochondrial fusion had a protective effect on MEFs treated with PES

To assess whether mitochondrial fusion protected cells challenged with PES, we relied on the Mdivi-1 compound as an inducer of mitochondrial fusion [18]. Following a pre-incubation of 6 hours with Mdivi-1 (40 μM), MEFs became significantly resistant to PES treatment (not shown). The resistance was even greater if Mdivi-1 was maintained throughout the treatment with PES after the pre-incubation period (Fig. 5A). The protective effect of Mdivi-1 was observed in MEFs containing BAX (wt MEFs) and those not expressing this protein (*Bax*-/- and DKO MEFs). However, the Mdivi-1 protection was significantly increased in wt compared to *Bax*-/- or DKO MEFs. To assess the effect of Mdivi-1 on mitochondrial fusion, wt MEFs were subjected to the Mdivi-1 pre-incubation and stained with a mitochondrial dye (MitoTracker®). Control, untreated cells displayed the spotted pattern expected if mitochondria are not fused (Fig. 5B). After 24 hours of Mdivi-1 treatment, a pattern of branched and filamentous mitochondria was observed (Fig. 5C). This pattern indicated an extreme condition of mitochondrial fusion. Finally, HeLa cells were also treated with increasing concentrations of PES, and a decrease in cell viability was observed. As reported above for the MEFs, HeLa cells were also protected from PES toxicity by the 6 hours of pre-incubation and maintenance with Mdivi-1 (Fig. 5D). In conclusion, the degree of mitochondrial fusion correlated with the protection of the cells treated with PES.

Discussion

The main purpose of this work was to assess the role of BAX and BAK proteins in the death process induced by PES. MEFs defective in BAX, BAK, both proteins and their wt counterparts were used as experimental tools. We concluded that neither protein was necessary for PES to cause cell death. Moreover, PES-induced cell lethality occurred without the activation of effector caspases. Therefore, apoptosis could be excluded as the mechanism of cell death triggered by PES in our model. Unexpectedly, BAX significantly protected cells

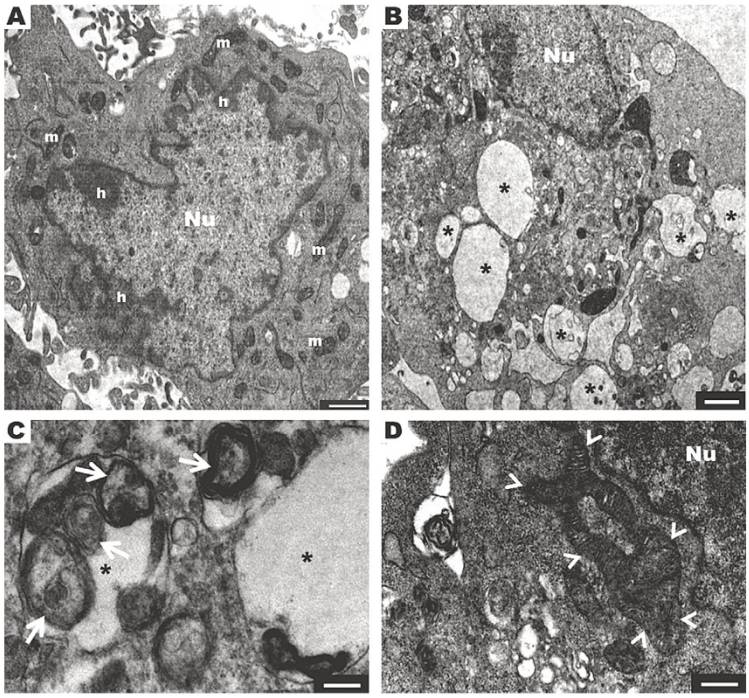

Fig. 4. Transmission electron microscopy of wt MEFs treated with PES. Control morphology of untreated wt MEFs. Nu: Nucleus, h: heterochromatin, m: mitochondria. Bar = 1.2 μm (A). Cell in an intermediate stage of the death process triggered by PES; the cell membrane remained preserved, but the cytoplasm was severely disrupted by vacuolisation, as indicated with asterisks. Nu: Nuclear chromatin. Bar = 1.8 μm (B). PES promoted the accumulation of autophagic vesicles (asterisks) with a characteristic multilamellar content (arrows). Bar = 0.16 μm (C). Fused and elongated mitochondria with preserved cristae were found after PES treatment (circumscribed by arrowheads). Bar = 0.36 μm (D).

from PES treatment. This result suggests that the cellular functions of BAX differ from those involved in mitochondrial outer membrane permeabilisation (MOMP) and apoptosis induction. Although somewhat unexpected, a pro-survival function for BAX has previously been reported. For example, hypoplasia due to cell death was observed in specific tissues in early studies of *Bax*-/- mice [19]. A survival effect for BAX was also observed in specific neuronal populations undergoing neurotrophic factor deprivation or Sindbis virus infection [20,21]. Finally, BAX overexpression reduced the ratios of cell death triggered by chemical compounds, such as Nigericin and 6-hydroxydopamine [22,23]. However, these reports do not provide consistent evidence about the nature of the pro-survival effect of BAX, and the issue remains very obscure.

We have shown that BAX needed to be inserted into the mitochondrial membranes in order to protect cells from PES treatment. Moreover, we demonstrated the ability of mitochondrial fusion caused by Mdivi-1 to counteract the toxic effects of PES. Based on these facts, we propose that BAX exerts a pro-survival effect via mitochondrial fusion. In support of our hypothesis, BAX is known to interact with the molecular machinery that controls mitochondrial fusion in healthy cells [24]. However, BAX is also located at the fission sites and involved in the extreme fragmentation of mitochondria observed in apoptosis [25,26]. Therefore, the involvement of BAX in mitochondrial dynamics is clear, but the final outcome seems controversial. Moreover, most members of the Bcl-2 family seem to participate in the regulation of mitochondrial dynamics, but a clear picture cannot yet be drawn regarding their precise involvement [27–29]. In Fig. 5A, we showed that Mdivi-1 more effectively prevented PES lethality when the BAX protein was present (wt MEFs), but BAX was not required for the protective effect of Mdivi-1 (*Bax*-/- or DKO MEFs). Mdivi-1 can likely promote mitochondrial fusion in the absence of BAX, although less efficiently. Finally, the protective effect of Mdivi-1 is not circumscribed to MEFs because it was also found in HeLa cells (Fig. 5D).

PES is believed to generate non-apoptotic, non-autophagic cell death by promoting proteotoxic stress [4,6]. In this context, we found BAX and mitochondrial fusion converging to prevent this type of cell death. In a previous report, BAX and mitochondrial fusion mediated by Mdivi-1 promoted necrotic cell death. In this report, ionomycin and the subsequent overload of cytoplasmic Ca^{2+} were the inducers of necrosis [30].. How can these opposite findings be conciliated? We propose that mitochondrial fusion generates opposite results depending on the death stimuli. The mitochondrial dynamics need to be studied further. For example, its role as a modulator of the cellular response to toxic insults awaits a systematic characterisation.

Normal T lymphocytes were found to be more resistant to PES than were leukaemic ones [9]. Similarly, non-transformed fibroblasts were far more resistant than the 10 tumour-derived cell lines tested in parallel [4]. The DKO MEFs studied herein are known for their resistance to chemotherapy. Therefore, they can be considered to display an aggressive oncogenic phenotype [31]. We have herein reported the increased sensitivity of *Bax*-/- and DKO MEFs to PES. The deficiency of BAX becomes a crucial factor to explain this increased sensitivity. In conclusion, we are expanding the profile of PES as a promising drug for its anticancer selectivity.

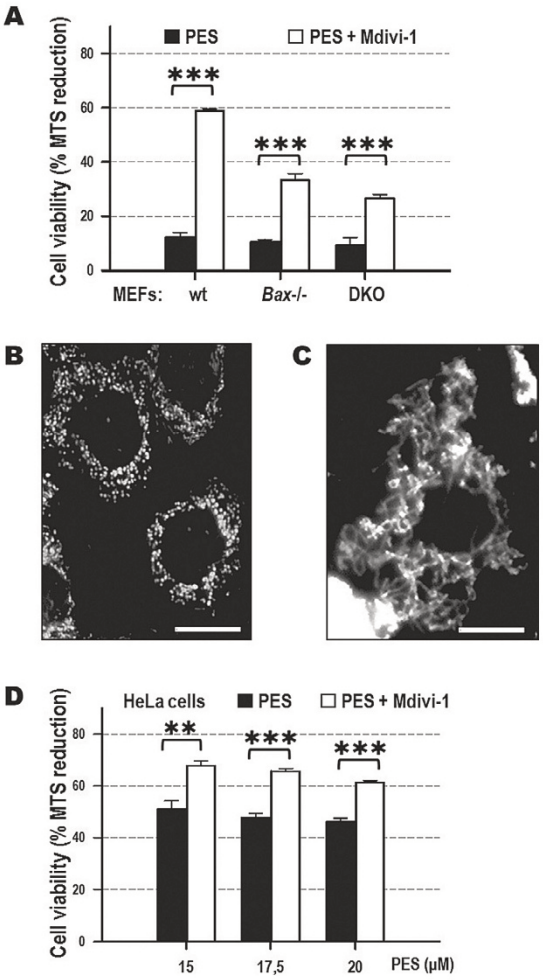

Fig. 5. Mdivi-1 induces mitochondrial fusion and cell resistance to death induction by PES. DKO, *Bax*-/- and wt MEFs were pre-incubated for 6 hours in Mdivi-1 (40 μM) (white bars) or DMSO vehicle (black bars). PES was then added for 12 hours, and the cell viability measured using the MTS procedure. The concentration of PES was 8 μM in *Bax*-/- and DKO MEFs. To equalise the cell viability rates in the absence of Mdivi-1, the concentration of PES was 12.5 μM in wt MEFs. The bar value is the mean ± SEM, n = 2, each n = 3 replicates. Student's t-test: $^{***}P < 0.001$ (A). Control untreated wt MEFs stained with MitoTracker® dye. Bar = 100 μm (B). Fused mitochondria in wt MEFs, incubated in Mdivi-1 (40 μM) and stained with MitoTracker®. Bar = 100 μm (C). HeLa cells were also pre-incubated for 6 hours with Mdivi-1 (white bars) or DMSO vehicle (black bars) as stated above. The cells were then treated with PES at the indicated concentrations, and the cell viability was determined. The bar value is the mean ± SEM of 3 replicates. Student's t-test: $^{**}P < 0.01$, $^{***}P < 0.001$ (D).

Specifically, cancer cells harbouring oncogenic BAX deletions or mutations are expected to preferentially succumb to PES.

Conflict of interest

No conflicts of interest are disclosed.

Acknowledgements

This work was supported by the Spanish Government, MINECO (Ministerio de Economía y Competitividad), project SAF2011-29730. Paolo Mattiolo was supported by an FI fellowship from AGAUR (Generalitat de Catalunya).

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4 Pharmacological Modulation of Reactive Oxygen Species in Cancer Treatment

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Current Drug Targets, **2015**, *16*, 31-37 31

Pharmacological Modulation of Reactive Oxygen Species in Cancer Treatment

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Abstract: Aerobic metabolism of mammalian cells leads to the generation of reactive oxygen species (ROS). To cope with this toxicity, evolution provided cells with effective antioxidant systems like glutathione. Current anticancer therapies focus on the cancer dependence on oncogenes and non-oncogenes. Tumors trigger mechanisms to circumvent the oncogenic stress and to escape cell death. In this context we have studied 2-phenylethinesulfoxamine (PES), which disables the cell protective mechanisms to confront the proteotoxicity of damaged and unfolded proteins. Proteotoxic stress is increased in tumor cells, thus providing an explanation for the anticancer selectivity of PES. In addition, we have found that PES induces a severe oxidative stress and the activation of p53. The reduction of the cell content in glutathione by means of L-buthionine-sulfoximine (BSO) synergizes with PES. In conclusion, we have found that ROS constitutes a central element in a series of positive feed-back loops in the cell. ROS, p53, proteotoxicity, autophagy and mitochondrial dynamics are interconnected with the mechanisms leading to cell death, either apoptotic or necrotic. This network of interactions provides multiple targets for drug discovery and development in cancer.

Keywords: Cell death, Cell experimental pharmacology, L-buthionine-sulfoximine, Mdivi-1, Oxidative stress, p53, 2-phenylethinesulfoxamine, proteotoxic stress.

BACKGROUND ON REACTIVE OXYGEN SPECIES

Free radicals are a concept of chemistry. A free radical is defined as an atom or molecule characterized by having unpaired valence electrons. Therefore it becomes a high-reactive entity with potential to interact and disrupt biological molecules, either the simplest (carbohydrates and lipids) or the most complex ones (nucleic acids and proteins). Free radicals are present in our environment as the result of chemical processes or ionizing radiation, for instance as a by-product of industrial activity. Alternatively, they can be produced *in vivo* as the result of the cellular metabolism.

Free radicals derived from oxygen are the most prominent in the cellular context. They are generated by the progressive reduction of molecular oxygen (O_2) to finally yield H_2O. In Fig. (**1A**), we show a classical scheme of this process plus the enzymes and reactions involved *in vivo* [1]. The ionic nature of superoxide anion ($O_2^{\bullet-}$) confers cell membrane impermeability to this entity. It is neutralized and converted to hydrogen peroxide by the superoxide dismutase enzymes (SOD). The hydroxyl radical ($OH^{\bullet}$) is highly reactive and, therefore, harmful for biological molecules. It is generated by the Fenton and Haber-Weiss reactions that require the participation of transition metals, frequently iron (Fe) in a biological context. Finally, hydrogen peroxide (H_2O_2) is no strictly a free radical. However, its oxidant capacity added to its efficiency in crossing biological membranes define a highly toxic profile for H_2O_2. In addition H_2O_2 becomes the precursor of $OH^{\bullet}$ radical via the Fenton and Haber-Weiss reactions mentioned above. These three molecules are collectively termed reactive oxygen species (ROS). The mitochondrial process of oxidative phosphorylation (OXPHOS) is the main source of ROS in the cells. The ATP yield of the OXPHOS process is greatly advantageous to eukaryotic cells but it implies intracellular toxicity by the generation of ROS. This ambivalent role of oxygen in life has been traditionally referred to as the "oxygen paradox". However, there are other locations where ROS can be generated. For example, there is a subtle and regulated release of ROS at the cellular membranes mediated by NADPH oxidases (Nox family of proteins) and involved in cell signalling [2].

Massive ROS generation is harmful for the cells. ROS oxidise and disrupt essential molecules like lipids, proteins and nucleic acids. Consequently cells counterbalance ROS by means of detoxifying enzymes or molecules with chemical reducing activity [1]. As shown in Fig. (**1A**), H_2O_2 is the substrate of catalase (CAT), peroxiredoxins (PR) and glutathione peroxidase (GPx). Vitamin E (VitE) and C (VitC) are molecules with reducing properties in specific intracellular compartments, lipophilic for VitE and hydrophilic for VitC (Fig. **1B**). The most relevant antioxidant molecule inside the cell is glutathione and this relevancy is even higher inside the mitochondrial matrix [3]. Because of its high intracellular concentration, reduced glutathione (GSH) determines the intracellular reduction potential. Once oxidised, GSH becomes glutathione disulphide (GSSG). Glutathione

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reductase (GR) is the enzyme that regenerates GSH. This reduction is coupled to the oxidation of NADPH (Fig. **1B**). GSH is a peptide composed of three amino acids, glutamate, cysteine and glycine. The enzyme glutamate-cysteine ligase (GCL), previously named γ -glutamylcisteine synthetase, catalyzes the first step in the synthesis of GSH. Then glutathione synthetase catalyses the binding of glycine and, as a result, glutathione (γ-glutamyl-cisteinylglycine) is generated. Glutathione (GSH) participates in many intracellular processes (Fig. **1B**). For instance it reduces the thiol groups of the oxidised proteins, the VitC and, indirectly, the VitE. GSH associates to GPx to transform H_2O_2 into H_2O. GSH can be conjugated to drugs, thus defining a specific type of phase II reactions in the metabolism of drugs. Finally, it can also be conjugated to proteins, thus becoming the post-translational modification termed glutathionylation [3]. In a healthy cell, ROS and antioxidant resources are in a homeostatic equilibrium, the imbalance leads to oxidative damage and, eventually, to cell death. The loss of the homeostatic balance is designated as oxidative stress.

CURRENT STRATEGIES IN CANCER TREATMENT

The beginning of the 21st century coincided with the commercialization of Imatinib mesylate (Gleevec®). Imatinib is an inhibitor of ABL tyrosine kinase. The function of ABL is to promote the survival of cells subjected to genotoxic stress. The reciprocal translocation that generates the Philadelphia chromosome is characteristic of chronic myeloid leukemia (CML). The translocation produces a fusion protein (BCR-ABL) and an abnormal increase of ABL activity. The cells of CML become dependent on ABL activity to circumvent cell death by apoptosis. Therefore, Imatinib triggers apoptosis of CML cells and an impressive clinical outcome in CML patients [4, 5]. Moreover, the specificity of Imatinib for ABL is not absolute. Imatinib also inhibits the c-KIT kinase that is relevant to the development of the gastrointestinal stromal tumor (GIST). Therefore, Imatinib has also become a successful treatment for GIST [6]. This type of therapeutic strategy has been designated as cancer specific, because the drug targets one or a few types of tumor exclusively, as exemplified by Imatinib. This strategy has also been labeled as personalized therapy of cancer because, for instance, among the patients with gastric cancer, only those with GIST are responsive to Imatinib. The common trait underlying this strategy is the dependence on one specific oncogene of one specific cancer cell (Fig. **2**). This evokes the phenomenon of addiction and, consequently, the "oncogene addiction" allegory has met some success to term this concept. Oncogenesis implies many disruptions in the cell homeostasis that incline cells to die, frequently via apoptosis. This cell predisposition uncovers a cellular stress that can be referred to as oncogenic stress. Therefore, the recruitment of antiapoptotic and other pro-survival mechanisms are needed for the progression and success of the oncogenic process. Actually, these pro-survival mechanisms become a druggable weakness, what has lead to the metaphor that "Achilles heels" exist in cancer cells.

Trastuzumab, a humanized monoclonal antibody against HER protein in breast cancer, Gefitinib and Erlotinib in those non-small cell lung cancers (NSCLC) with mutated EGF receptor, or Crizotinib for NSCLC with the fusion protein EML4-ALK, are examples of drugs directed to the "Achilles heel" of specific types of tumors, in clinical use presently [7]. Still in an experimental phase, the possibility of a combined drug therapy emerges. For instance, the synergistic association of ABT-737 and Roscovitine or its R-enantiomer (Seleciclib) [8, 9]. Many tumor cells are dependent on the antiapoptotic *Bcl-2* gene to circumvent the oncogenic stress. This stress can be caused by the increase of some of the BH3-only proteins, which are pro-apoptotic and antagonistic of Bcl-2 (Fig. **2**). In this context, specific inhibitors of Bcl-2 like ABT-737 are of great interest. However, it has been found that cells become resistant to ABT-737 by overexpressing Mcl-1, a short-lived, antiapoptotic protein of

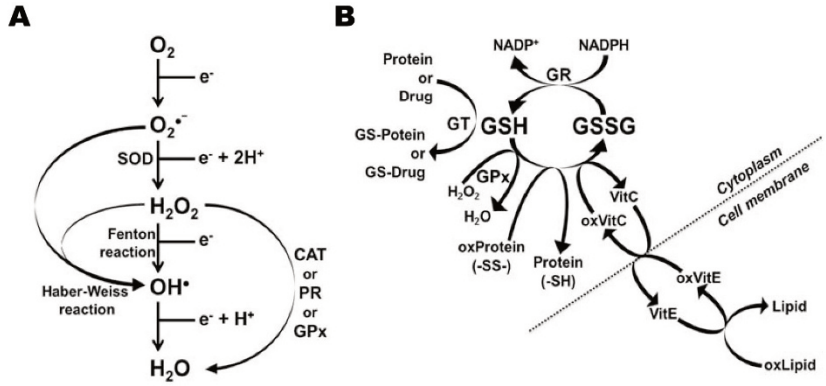

Fig. (1). (A) Graphical summary of the chemical process of O_2 reduction to H_2O. The reaction catalysed by superoxide dismutase enzymes (SOD) is shown. The generation of hydroxyl radical ($OH^{\bullet}$) by Haber-Weis and Fenton reactions is indicated. Finally, catalase (CAT), peroxiredoxin (PR) and glutathione peroxidase (GPx) are the enzymatic activities devoted to neutralize hydrogen peroxide (H_2O_2) inside the cells. **(B)** Scheme of the glutathione (GSH) redox cycle and its coupling to other redox cycles and conjugation reactions inside the cell. The ox- prefix denotes the oxidized form. Glutathione reductase (GR), glutathione peroxidase (GPx), and the superfamily of glutathione transferases (GT) are indicated close to the reactions they catalyse.

the Bcl-2 family. Mcl-1 is not inhibited by ABT-737. In this context, the inhibition of CDK9 and the subsequent transcriptional elongation phenomenon by Roscovitine/Seleciclib promotes an early and fast decay of the Mcl-1 protein [10]. Therefore, ABT-737 and Roscovitine/Seleciclib can be successfully combined to kill cancer cells selectively (Fig. **2**).

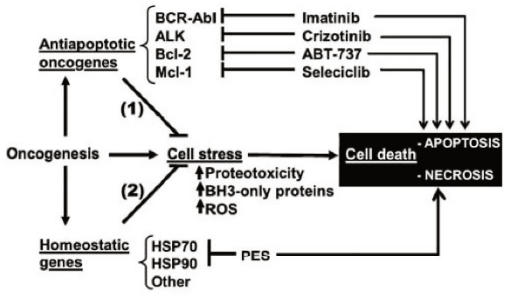

Fig. (2). Scheme illustrating how the oncogenic process sets the cells in a state of stress that predisposes to cell death, for example by an elevated ROS content and oxidative stress. To avoid their demise, cells follow two different strategies: (**1**) Oncogene dependence. (**2**) Non-oncogene dependence. This allows the identification of targets for drug development. This approach is supported by the success of drugs such as Imatinib, Crizotinib and other, presently and routinely used in patient treatment.

Stressed cells rely on homeostatic mechanisms such as the chaperone activity of heat shock proteins (HSP). The same holds true for tumors that are naturally exposed to oncogenic stress. These homeostatic mechanisms are not intrinsically oncogenic but a response to the oncogenic stress and, therefore, the concept of non-oncogene dependence or "non-oncogene addiction" of cancer can be proposed (Fig. **2**). The best examples of drugs following this strategy are the inhibitors of HSP70 (PES) and HSP90 (Geldanamycin). HSP70 and 90 prevent misfolding and aggregation of proteins due to their chaperone activity. In addition, HSP70 is involved in the autophagic clearance of proteins either misfolded or aggregated. PES (2-phenylethinesulfonamine) has proven to be quite selective at killing cancer cells by promoting proteotoxic stress and causing a necrotic type of cell death [11, 12]. This fact has stimulated the research in this category of innovative drugs [13-15].

There are other cellular phenomena that can be harnessed to fight cancer. Cells can be induced to terminally differentiate or to enter senescence. Both processes imply a cytostatic effect, i.e. cell quiescence and the stop of the cancer growth. Nevertheless killing cancer cells, i.e. a cytocidal effect, seems the most direct approach to achieve cancer regression. There are different types of cell death that are periodically classified and catalogued [16]. In brief, type 1 is apoptosis, which morphological and molecular definition is precise. Type 2 is autophagic cell death, which is considered a misnomer. Autophagy is frequently found in dying cells, but it is rarely involved in causing the cell demise. On the contrary, autophagy is essentially helping the cells to get rid of protein aggregates, damaged organelles and subsequent ROS production. Type 3 is necrosis, which is characterized by membrane disruption and the spillage of the cell content. Because of this spillage, necrosis has the ability to promote immunity, strong inflammatory responses and tissue disruption. This fact has traditionally been considered negative when compared to the silent, self-contained apoptotic process. However, apoptosis is not so self-contained. Some apoptotic cells expose Calreticulin at the cell surface and secrete ATP and HMGB1 protein. These events are highly immunogenic and have proved to be crucial for succeeding in causing tumor regression with apoptosis-inducing agents. This is the case of Doxorubicin, Cyclophosphamide, Bortezomib or γ - irradiation [17, 18]. Accordingly, the interest of the agents that induce necrotic cell death should be reconsidered.

OXIDATIVE STRESS, P53 AND CANCER THERAPEUTICS

In Fig. (**2**), elevated ROS are part of the cellular stress derived from oncogenesis. In other words, oxidative stress becomes part of the oncogenic stress of a cell. Is this true for all cancer cells? Is this an "Achilles heel" to be exploited in the treatment of a few, many or most tumors? The answers are subject to controversy. For instance, inactivating mutations and deletions of p53 are the most common event in human cancer [19, 20]. As a tumor suppressor, p53 senses many different stresses of the cell and triggers a plethora of responses, ranging from cell cycle stop to apoptotic cell death [21]. In spite of being a transcription factor, p53 can activate either transcriptional or non-transcriptional responses [22]. For instance, its translocation to mitochondria to directly activate the mitochondrial outer membrane permeabilisation (MOMP) and apoptosis [23]. The search for a drug with the ability to block this translocation was the aim of the research that led to the discovery of PES. This fact explains the alternative designation of PES as pifithrin-μ [24]. Among the genes regulated by p53 is TIGAR (TP53-induced glycolysis and apoptosis regulator). This gene connects p53 and the regulation of glucose metabolism and ROS. TIGAR promotes the redirection of glucose towards the pentose phosphate shunt and, therefore, increases the production of reduced NADPH [25]. Consistently, a reduced function of TIGAR provides a good explanation for the increased ROS phenotype of tumor cells with a defective function of p53. In this context, the ROS threshold hypothesis for cancer therapy was formulated. In brief, tumor cells suffer from oxidative stress to a greater extent than non-tumor ones and, as a consequence, they are closer to the threshold of cell death induction by ROS. Accordingly, the therapeutic agents with the ability to increase ROS will preferentially harm the tumor cells [26]. Indeed, broadly-use therapies such as irradiation and anthracyclines act, in part, by increasing ROS in cancer cells. Particularly, the glycopeptide bleomycin has a mechanism of action directly based on ROS generation and subsequent DNA damage and fragmentation. The recently approved arsenic trioxide is also a direct producer of ROS in cancer cells. Regarding cancer therapy, several potential new drugs with the ability to promote oxidative stress have reached clinical phases of development (Table **1**).

None of the above mentioned compounds displays the type of anticancer specificity observed in the treatments with Imatinib, Crizotinib, etc. They are promising agents but be-

have as non-selective chemotherapeutic drugs. There are two important caveats concerning this therapeutic approach. First, cancer cells are heterogeneous inside a tumor and, as a consequence, some cells can be far away from the lethal threshold of ROS. Second, the hypoxic and nutrient-poor tumor environment selects for cells with increased antioxidant capacity, i.e. elevated GSH content. This GSH-rich phenotype is associated with the proficiency of the cancer cells to metastasize [28]. In conclusion, a ROS-inducing treatment could be overtly partial and promote a more aggressive phenotype. In our opinion, the possibility to be specific or succeed in cancer treatment simply by increasing ROS is scarce. However, we are not so reluctant about the possibility to combine drugs that encompass oxidative stress, as we will discuss below.

Table 1. ROS modulating agents undergoing clinical trials in oncology [27].

Agent	Mode of Action
NOV-002	A form of oxidised glutathione (GSSG) that promotes intracellular GSH/GSSG imbalance, therefore mild oxidative stress and protein glutathionylation
BSO (L-buthionine-sulfoximine)	Inhibitor of glutamate cysteine ligase (GCL) that causes the synthesis of GSH to be impaired. In this inhibition context, GSH can be depleted by oxidative stress
Canfosfamide	Inhibitor of glutathione transferase (GT) activity
Ezatiostat hydrochloride	Inhibitor of glutathione transferase (GT) activity
Imexon	Pro-oxidant molecule able to deplete cells from GSH
Disulfiram	Acetaldehyde dehydrogenase inhibitor, classical inducer of alcohol intolerance and pro-oxidant of GSH
PX-12	Inhibitor of thioredoxin-1, which is overexpressed in tumors with an aggressive phenotype
Dimesna	Dual inhibitor of thioredoxin-1 and glutaredoxin by an ill-defined mechanism
Motexafin gadolinium	Dual inhibitor of thioredoxin reductase and ribonucleotide reductase
Darinaparsin	Arsenic derivative

Initially observed in 1924 by Otto Warburg, the Warburg effect has lately attracted a great attention in oncology [29]. The effect consists in the shift of glucose metabolism from OXPHOS to lactate production in an oxygen-rich context. Therefore, it has been described as aerobic glycolysis. This phenomenon has provided the basis of an imaging technique for the diagnosis and follow-up of some tumors. This technique comprises cell labelling with ^{18}F-deoxyglucose and positron emission tomography (PET) scanning. Unfortunately, to date, no similar applications have succeeded in the area of cancer therapy. One obvious question is how the Warburg effect impinges on the intracellular ROS content. The answer is that the amount of ROS generated by the OXPHOS process will be greatly reduced. Therefore, the Warburg effect does not help to explain why many cancer cells display increased ROS. Moreover, it seems to oppose the drugs that induce oxidative stress thus easily explaining the development of resistance. Finally, as mentioned above, it can be associated with the GSH-rich phenotype found in metastasis.

The relationship of p53 and ROS is paradoxical because, in opposition to TIGAR, some of the genes regulated by p53 generate ROS. This is the case of *PIG3* (quinone oxidoreductase) and *PIG6* (proline oxidase). Under mild p53 activation, cell cycle stop and antioxidant activity (TIGAR) would be promoted to ease the reparation of the damage in cellular DNA. This is consistent with the antioxidant role of the Warburg effect, that has been conceived as a strategy to increase the efficiency of DNA replication [29]. Conversely, under strong p53 activation, cell death and ROS would prevail [30]. The possibility to reactivate p53 in cancer cells has traditionally been approached with great interest [31]. Some drugs act by counteracting some inactivating mutations of p53. Others, like Nutlin-3, increase p53 activity in tumors like human neuroblastoma that are usually characterized by a preserved p53 response [32]. Drugs acting as agonists of p53 will be expected to cause MOMP, apoptosis and ROS production. On the other hand, the generation of ROS will cause DNA damage and p53 activation. In conclusion, ROS and p53 are engaged in a positive feed-back loop. Moreover, ROS can trigger either MOMP and apoptosis or the opening of the mitochondrial permeability transition pore (MPTP) and necrosis. Both, apoptosis and necrosis are inducers of ROS. Again, this is a positive feed-back that drives the cells to their demise. The phenomenon of MOMP is under the control of the Bcl-2 family of proteins, consequently the pharmacological modulation of this event is possible by means of drugs like ABT-737, which neutralises some of the antiapoptotic members. Finally, in some experimental paradigms, p53 is involved in triggering necrotic cell death and ROS are clearly involved [33]. Taken these facts altogether, a complex net of mutual interactions can be considered (see Fig. **5**).

CELL DEATH INDUCTION BY THE ASSOCIATION OF PROTEOTOXIC AND OXIDATIVE STRESS

The screening of chemical libraries to find specific inhibitors of the translocation of p53 to mitochondria allowed the discovery of PES [24]. Consistently, PES protected cells from some death stimuli mediated by this translocation. Surprisingly, PES proved to be very lethal for neoplastic cells [11, 12]. PES is a small molecule with chemical traits for being a highly reactive and oxidant agent (Fig. **3A**). A characterization of its mode of action revealed its ability to block HSP70 function *in vivo* and as a consequence: i) the secondary inhibition of HSP90 and the proteasome; ii) the blockage of the chaperone-mediated type of autophagy, which results from HSC70 inhibition; iii) the impairment of the completion of macroautophagy (named autophagy hereafter) [12, 34]. The implications for the cells are severe because

four pivotal mechanisms to eliminate misfolded and aggregated proteins are lost simultaneously. We have consistently observed how multilamellar structures accumulate in the cells treated with PES, as the result of blocking the autophagic flux and the subsequent lack of autophagosome clearance [35, 36]. The process ends up in a necrotic phenotype with the rupture of the cell membrane [36]. At earlier stages we can see the depositions of a proteinaceous material, named aggresomes, which indicates damaged proteins in aggregated clumps (Fig. **3B**). We can also observe different stages of mitochondrial damage, i.e. a variable degree of cristae disruption in swollen mitochondria (Fig. **3B**). In conclusion, proteotoxic stress is a clear outcome of PES treatment but the downstream mechanisms leading to cell death remain ill-defined. The hypothesis about cancer cells being closer to the lethal threshold of proteotoxicity than normal ones provides a good explanation for the higher susceptibility of cancer cells to PES. Consistently, we have experimentally determined that cells genetically deficient in autophagy are more sensitive to PES (unpublished results). Undoubtedly, the deficit in autophagy raises the proteotoxic stress in these cells.

The cellular effects of PES were consistent with an early induction of ROS, which was reversed by a reducing agent like N-acetylcysteine (Fig. **3C**). This reversion translated into an increased cell survival at longer times of treatment [36]. The quantification of intracellular ROS is routinely performed by the reagent 2',7'dichlorofluorescindiacetate (DCF-DA). The compound enters the cells and is processed to DCF by the intracellular esterases, thus becoming captured inside the cell. Then DFC fluoresces upon oxidation and in proportion to the amount of intracellular ROS. In conclusion, a remarkable oxidative stress was involved in the mode of action of PES. This fact is important because ROS react with proteins and cause proteotoxic stress. The relative amount of proteotoxicity caused by ROS or, alternatively, by HSP70 inhibition in a cell treated with PES is unknown and not easy to discern. The concentrations of PES required to inhibit HSP70 *in vitro* are higher than those required *in vivo*. Therefore, the molecular details of HSP70 inhibition by PES remain unclear [37]. The early rise of ROS supports our speculation about a role for PES-triggered oxidative stress in HSP70 inhibition *in vivo*. In addition we have demonstrated that p53 is involved in the necrotic cell death triggered by PES [36]. Moreover, we conclude that a positive feed-back between ROS and p53 is leading the cells to their demise.

BSO (L-buthionine-sulfoximine) is a small molecule with an amino acid structure (Fig. **4A**). BSO is a pharmacological inhibitor of the enzyme GCL and, therefore, it is able to block the synthesis of GSH in the cells. As a consequence, the cells loose their reduction potential and become more susceptible to oxidative stress. The combination of PES and BSO potentiates the generation of ROS (Fig. **4B**). The combination of PES and BSO is synergic at inducing death in several cell lines [36]. This synergism provides another evidence of the involvement of ROS in the PES mode of action. Furthermore, it suggests a therapeutic opportunity. BSO is characterized by being minimally toxic for cells in culture unless other cellular insults coincide. Consistently, in clinical trials, BSO shows minimal toxicity for humans [38]. The association of BSO and conventional chemotherapy has been investigated in the past. For instance, in ovarian cancer BSO shows synergism with melphalan and cisplatin [39, 40]. Similarly, in colon and hepatic carcinoma, BSO and azathioprine synergise [41]. In spite of these reported synergisms, BSO has still not reached clinical use. The synergism of BSO and PES is promising. It will allow the reduction of the doses of PES without reducing the ratios of cell death. Undoubtedly, a reduced dose of PES will translate into minimising its side effects. However, many questions remain. Will the tumor selectivity of PES be maintained in association with BSO? What are the toxic effects of PES in humans? How severe can these side effects be? Notwithstanding these questions, published evidence supports the interest of combining two drugs that cause proteotoxic and oxidative stress respectively [42].

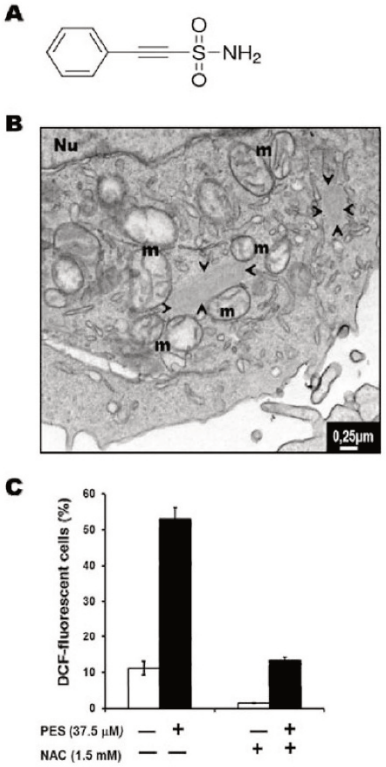

Fig. (3). (A) Chemical structure of 2-phenylethynesulfonamide (PES), named also Pifitrin-µ. **(B)** Transmission electron microscopy of HCT116 cells treated for 48 hours with PES (25 µM). Nuclear chromatin (Nu). Mitochondria at successive stages of cristae disruption (m). Deposits of proteinaceous material in the cytoplasm defined as aggresomes (delimited by arrowheads). **(C)** HCT116 cells were treated as indicated in the graph with PES, the antioxidant N-acetylcysteine (NAC) or the combination of both for 1 hour. ROS detection by means of the DCF-DA reagent was performed. Cells were incubated with DCF-DA, treated afterwards and flow cytometry was finally used to quantify the percentage of cells that were fluorescent by containing the oxidised DFC. The bar values are the mean ± SEM of several independent determinations.

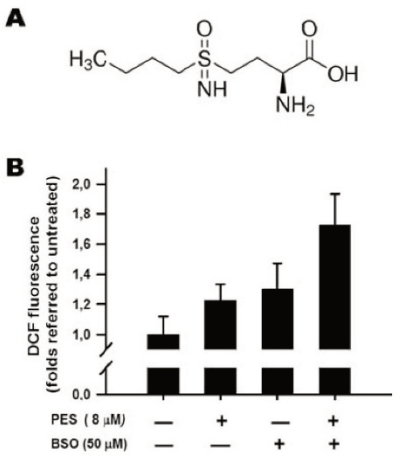

Fig. (4). (A) Chemical structure of L-buthionine-sulfoximine (BSO). **(B)** U87MG cells were treated as indicated in the graph with PES, BSO or the combination of both for 6 hours in 96 multiwell plates. ROS detection was performed by the DCF-DA procedure, as before. However, the fluorescence of oxidised DFC was recorded by means of a multiwell plate reader. The readings were referred to the untreated condition, which was assigned the unit value. They-axis is expressed as folds over the unit value. The bar value is the mean ± SEM of 5-6 determinations.

In our studies on PES, we found the paradoxical result of Bax protein displaying a pro-survival function [35]. Bax and Bak are pro-apoptotic members of the Bcl-2 family of proteins. Both proteins are pivotal elements in the MOMP that leads to apoptosis. Bax is known to be involved in the regulation of mitochondrial dynamics, i.e. the state of fusion or fission of mitochondria [43]. This fact prompted us to explore this issue. As a tool we used the compound Mdivi-1, which promotes mitochondrial fusion. Mdivi-1 is an inhibitor of the GTPase activity of DRP-1, the protein in charge of the mitochondrial fission process [44]. We found that mitochondrial fusion had a protective effect in cells treated with PES [35]. It is known that mitochondria respond to oxidative stress by fusion. Mitochondrial fusion is interpreted as a mechanism to mix the contents of healthy and damaged mitochondria. This allows the compensation of the damaged content, such as oxidised mitochondrial DNA, by undamaged one [45]. Our results are coherent with this explanation since PES was inducing oxidative stress and the promotion of mitochondrial fusion was mitigating the toxicity of PES.

In conclusion, we envisage ROS as a converging node susceptible of direct (BSO) and probably indirect (PES) pharmacology. We have previously commented the positive feed-back between ROS and p53, ROS and apoptosis, and ROS and necrosis (Fig. **5**). PES illustrates another positive feed-back between proteotoxicity and ROS. On one hand, ROS causes chemical modifications, subsequent misfolding and aggregation of proteins. On the other hand, proteotoxic stress associated to PES translates into oxidative stress. Moreover, the failure of autophagy is also a facet of the PES mode of action. Autophagy is considered a mechanism of cell defence. Autophagy alleviates cells from protein aggregates and damaged mitochondria. Finally, mitochondrial dynamics adds another dimension to the scheme. Mitochondria fuse to minimise the effects of ROS. However, giant fused mitochondria elude autophagy. Apoptosis is associated to mitochondrial fission by stimulating the complete proteolysis of the protein OPA-1 and OPA-1 is necessary to accomplish the fusion of the inner mitochondrial membrane [45]. Taken these facts altogether, a complex network of interactions is envisaged (Fig. **5**). This scheme indicates several targets to be investigated by experimental pharmacologists. In addition, the network summarises the mode of action of PES and highlights the involvement of ROS.

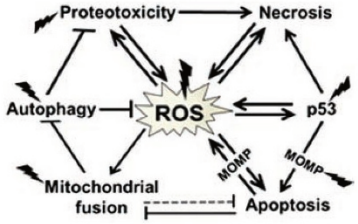

Fig. (5). Scheme illustrating how ROS are a central node that stablish feed-back positive loops with protetoxic stress, p53 and the mechanisms leading to cell death, either apoptotic or necrotic. Mitochondrial outer membrane permeabilization (MOMP) is mediating apoptosis and under regulation by the Bcl-2 family of proteins. Based on our results, the participation of autophagy and mitochondrial dynamics (state of mitochondrial fission or fusion) has been included in the network of interactions. The existence of drugs targeting these events, like PES, is symbolized by thunderbolts. These drugs are potential subjects for experimental pharmacology of cancer. Activation is indicated by arrows. Inhibition is depicted as a bar capped line. The dashed line means no conclusive evidence.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Research in the Pharmacology Unit is funded by project SAF2011-29730, MINECO (Ministerio de Economía y Competitividad), Spanish Government. Paolo Mattiolo is supported by a FI fellowship from AGAUR (Generalitat de Catalunya).

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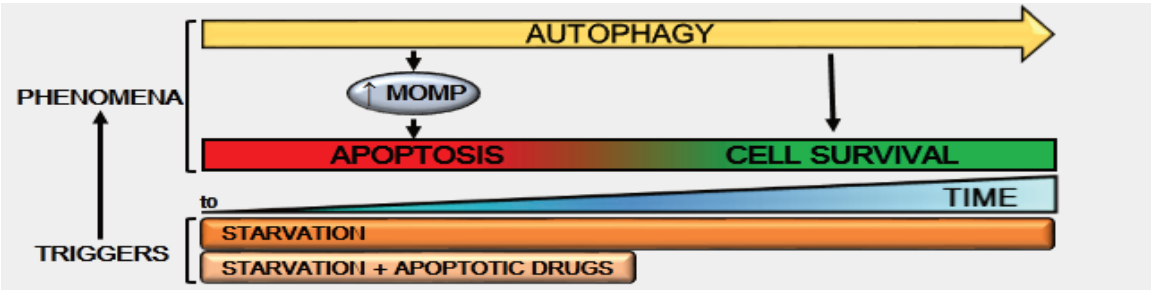
Received: September 16, 2014 **Revised: October 29, 2014** **Accepted: November 07, 2014**

PMID: 25395102

VII DISCUSSION

1 Discussion paper 1

“Autophagy exacerbates caspase-dependent apoptotic cell death after short times of starvation”



Our experiments uncovered a pivotal role of autophagy in priming caspase-dependent apoptotic cell death upon growth factor, glucose and nutrient deprivation (starvation media, SM), while triggering cell survival at long times. Our results put together both outcomes under one single trigger: starvation.

Mechanisms of SM-induced cell death tolerance

Within a solid tumor, cancer cells undergo transient periods of starvation that translate into a gradient of metabolites (glucose, non-essential aa, etc.) oxygen and growth factors from the tumor core to the external and more irrigated layers of the cancer mass. The ability to stimulate angiogenesis but also to tolerate metabolic stress is a critical factor for cancer survival and progression. The cell tolerance to different types of starvation is a matter of study. For instance, deprivation of a single component at a time is a standardized experimental approach, but can lead to compensatory metabolic readjustments, especially if certain nutrients are depleted. In our studies, we employ starvation media (SM), which is one of the most severe starvation forms (no glucose, no nutrients and no growth factors). Under this condition, we avoid any possible compensatory effects on metabolism. This environment induces cells death (and autophagy) in all the analyzed cell lines (MEFs, PC3, SH-SY5Y, MCF-7, HeLa, DU145, HEK293, U87MG, HCT116). WT MEFs, an immortalized non-tumor cell line, was the most sensitive to SM-driven cell death. This characteristic, in addition to the availability of several isogenic knock-out cell lines, made them a suitable model for our experiments. In this cell line, SM-induced cell death was alleviated by the addition of many single metabolites. Glutamine and glucose, two of the most frequent energetic sources for a cell, exerted a protective effect facing SM-driven lethality. For instance, WT MEFs subjected for 24h to SM plus 250 µM of glutamine were completely protected facing cell death. Similarly, 2 g/L of glucose markedly reduced caspase activation and mitigated cell death (data not shown). Interestingly, WT MEFs cells underwent starvation-driven cell death at times earlier than 24h. In sharp contrast, other cell lines (both tumoral and non-tumoral) were able to survive starvation for longer times. There are different hypothesis to explain the metabolic tolerance of some employed cell lines to SM-driven cell death:

- **Increased β-oxidation is responsible of SM-resistance.** Cells subjected to SM undergo metabolic changes aimed to maintain a functional level of ATP. In this setting, ATP can be obtained from energy stores (glycogen, lipids droplet) and metabolic intermediates recycled through autophagy. Increases in the AMP/ATP ratio are sensed by AMPK. Upon its activation, AMPK reestablishes the energy levels by inhibiting anabolic and enhancing catabolic pathways (e.g. β-oxidation) [89]. We have several evidences that support the activation of AMPK in our models. For instance, cells treated with SM plus 250 µM AICAR (an analog of adenosine monophosphate that acts as an activator of AMPK) display a reduced caspase activity. On the opposite, the pharmacological inhibition of β-oxidation (Etomoxir, TOFA, Cerulenin and C75) exacerbates SM-driven cell death in all the employed cell lines (data not shown). Thus pointing out to the lipid β-oxidation as a central player in the SM-driven cell tolerance.

- **SM-induced cell death is dampened by hyperactive prosurvival pathways in cancer cell lines.** In normal proliferating cells, nutrient uptake and metabolism are tightly regulated by growth factors. The growth factors (GF) signaling cascades converge mostly on the RAS/ERK or PI3K/AAK pathways. As a consequence, growth factors (GF) trigger a plethora of effects, such as cellular growth, cell survival and metabolic reprogramming [110]. Multiple cancer subtypes undergo alterations of RAS and AKT cascades that act as permissive events for the tumorigenic process. In normal cells, long periods of glucose deprivation trigger the activation of Akt [111], while serum deprivation leads to the inactivation of the PI3K/AKT pathway [112]. Despite we do not have information on the status of the AKT pathway in the studied cell lines, it is possible to surmise that the presence of an abnormal activation of these oncogenic pathways may contribute to prolonged cell viability by dampening apoptosis and promoting the cell survival. In this sense, high levels of AKT in pancreatic cancer cell lines seems to participate in the resistance facing a complete starvation without being a requisite [113].

- **Secreted paracrine factors promote survival of cultures exposed to SM.** *In vitro,* cells can condition the culture medium by releasing a series of protein, lipid, sugar or oligonucleotide-based factors. Secretion can occur through classical (i.e. ER/Golgi-dependent manner) and a non-canonical secretory pathways (i.e. shedding of plasma microvesicles, direct efflux through plasma membrane and exosomes) [114]. As mentioned in the previous section, these products could converge on the activation of pro-survival pathways such as RAS/ERK and PI3K/AKT. In this sense, cancer-associated fibroblasts undergoing autophagy release metabolic intermediates (lactate) to sustain the tumor growth [115]. This phenomenon has been termed as the reverse Warburg effect. Thus being one of the best proof-of-concept to the proposed hypothesis.

- **Engulfment of apoptotic bodies from a subpopulation of SM-driven apoptotic cells contributes to the survival of a remaining starved subpopulation.** Cancer cells are able to engulf dying or alive neighbouring cells in a process generally termed cell engulfment [116, 117]. Different forms of cell engulfment exists, among which cannibalism, phagocytosis, entosis. Phagocytic activity has been for example observed in cancer cells line such as MCF-7 [118], while cannibalism has been reported in bladder cancer (transitional cell carcinoma), breast cancer, lung carcinomas. This acquired capacity represents a sign of the aggressiveness of the tumor and, in condition of deprivation, another means to fuel the metabolism of part of the culture. SM-Resistant cells may feed on apoptotic bodies from their weaker neighbouring cells or, alternatively, eat "marked" alive cells. The factors playing a role in this process remain an incognita.

<u>Pro-death role of autophagy at short-times of SM</u>

Cultures subjected to starvation media (SM) display clear signs of autophagy and activation of the caspase-dependent intrinsic apoptotic pathway. This phenomenon has been reported in several experimental paradigms [119, 120, 121, 122].

Autophagy-incompetent cells (*Atg 5* $^{-/-}$ and 3-MA treated cells) shown less cyt C release and an attenuated caspase activation, and thus they are protected facing SM-driven apoptosis. Based on these observations, we concluded that at short times, autophagy acts as a mechanism of MOMP-dependent apoptosis. On the contrary, bafilomycin A_1 – an inhibitor of the late stages of the autophagic pathway – was unable to confer cytoprotection at the same time points (data not shown). These results, suggest that the engagement of the autophagic machinery is crucial for the induction of MOMP and exclude the involvement of mechanisms initiated at the autophagolysosome stage. Furthermore, autophagy-incompetent cells (*Atg 5* $^{-/-}$ and 3-MA treated cells) subjected to SM are protected facing a series of apoptotic compounds (STP, Etoposide, Camptothecin). Altogether, these results corroborate that the autophagic machinery - at least it the context of nutritional and trophic deprivation – is promoting spontaneous or pharmacologically-induced apoptotic cell death. The literature offers several possible explanations to this phenomenon based of a complex regulatory network between BCL-2 family members and autophagy. These are the hypotheses we favor the most:

- **The autophagy-dependent sequestration of anti-apoptotic BCL-2 family triggers the intrinsic pathway of apoptosis.** The sequestration of anti-apoptotic proteins within the autophagosomes could occur through selective or not selective mechanisms. A plethora of poly-ubiquitin chain binding proteins have been identified. For instance, p62 can selectively target a series of specific proteins to the autophagosomes during CMA. Nonetheless, GATA4, a transcription factor involved senescence and inflammation is the first protein to be specifically-targeted to the autophagosomes via p62 [123]. Finally, the unbalance between mitochondrial pro-apoptotic and anti-apoptotic proteins will trigger the MOMP.
- **SM-driven upregulation of Beclin-1 favors apoptosis by promoting the clearance of MCL-1.** This hypothesis is supported by the recent observed inverse correlation between levels of MCL-1 and Beclin-1, a phenomenon responding to their reciprocal regulation due to a competition for a common deubiquitinase - USP9X - [124]. In our setting, we observed an increase in the level of Beclin-1 (data not shown). Accordingly, such increase would lead to an enhanced degradation of MCL-1, thus prompting the intrinsic apoptosis pathway.
- **Autophagy upregulates BH3-only proteins**. MOMP is positively regulated by a series of sensitizing and activating pro-apoptotic proteins. The proposed hypothesis would imply an autophagy-dependent upregulation of at least one BH3 only protein. To our knowledge, there is no experimental evidence of autophagy playing this role. On the other hand, as described in section **5.3.4,** the apoptotic-driven cleavage of specific autophagic proteins generates truncated fragments, containing a BH3 domain. For example, caspase-3, -6 and -9 dependent cleavage of Beclin-1 generates a truncated C-terminal fragment, which prompts the release of pro-apoptotic factors from isolated mitochondria. In a similar manner, the calpain-mediated cleavage of ATG5 generates an N-terminal 24 kDa fragment that interacts with BCL-X_L sensitizing cells to apoptotic cell death. In our setting, Calpeptin (a calpain specific inhibitors) wasn't able to exert any significative protection at short times, thus indirectly excluding this possibility (data not shown).

Pro-survival role of autophagy at long times of SM

At long times of treatment, our results unveil a pro-survival function of autophagy. After 12h SM, Autophagy-incompetent cells (3-MA treated and *Atg 5* $^{-/-}$) exhibit a greater cytotoxicity (Paper 1, Fig. 5D) than the autophagy-proficient ones. Surprisingly, this event occurs despite a similar or greater release of cyt C (Paper 1, Fig 3A, 3B, 4B) and diminished caspase activation (Paper 1, Fig 3C, 3D, and 4C). This implies that SM-driven autophagy undergoes a shift of function determined by time. Blockage of autophagy under most deprivation models is a well-known trigger of cell death [125]. An exception to this rule is the glucose starvation paradigms. Under this condition, autophagy is not activated and has no reported protective effect [126]. In our setting, time would control the change between the pro-death/survival effects of autophagy. These are the hypothesis we favor the most to explain the switch of function of autophagy towards cell survival:

- **Time controls the appearance of the pro-survival function of autophagy.** We propose that over time, the pro-apoptotic effect of autophagy is overcome by its protective function. This could be cause by different possible mechanism which requires a certain time to occur. Autophagy could promote the expression of unknown prosurvival factors, the recycling of nutrients or the specific induction of mitophagy. Mitophagy is for instance, described to limit the release of pro-apoptotic factors though the clearance of damage mitochondria, as reported in other paradigms [119].

- **Autophagy promotes cell survival through the suppression of necrotic cell death.** Necrosis and apoptosis represent the two extremes of a continuum of cell death subroutines [33]. Changes in specific biochemical parameters (e.g. ATP, antioxidant levels) or in the magnitude of a specific stress can lead to a conversion of apoptosis to necrotic cell death [127]. In light of the discrepancy between caspase activation and cell death, we surmise that prolonged SM treatment will engage caspase-independent (necrotic) cell death. We favor the following models to explain the involvement of necrosis in our setting:

- **Energy based-model:** ATP is used as the most frequent energy molecule within a cell and accordingly, sustaining sufficient ATP levels is a capital event for cell survival. A decrease in the ATP content is a powerful signal to trigger cell death. The kinetics of ATP collapse are determinant in the commitment to either apoptotic or necrotic cell death [128].
  In our model, necrosis could occur in a subpopulation of cells already succumbing by the intrinsic apoptotic cell death. In this sense, progressive energy withdrawal would lead to a greater proportion of cells being committed to necrotic, rather than apoptotic death [129]. In SM, the switch from apoptosis to necrosis would befall downstream of the apoptosome stage, as a consequence of the first phase of autophagy-dependent MOMP with loss of mitochondrial potential ($\Delta\psi_m$) and subsequent bioenergetics catastrophe.

  Alternatively, necrosis could occur in an apoptotic-resistant subpopulation that survives the initial metabolic stress. In this case, the ATP level collapse would be a consequence mainly of an exhaustion of substrate to catabolize. This possibility is supported by the experiments in the isogenic *Bax* and *Bak* (DKO) knock-out MEFs. DKO MEFs display complete protection facing SM for 24h and succumb in the following 48h by necrosis as proved by the absence of caspase activation up to 72h (data not shown). This results corroborates the induction of necrotic cell death in DKO MEFs at long times of SM treatment and suggest that the same phenomenon could be occurring before 24h of treatment in WT MEFs. In addition, the lack of caspase activity excludes the involvement of caspase-8-dependent apoptotic cell death in our experimental paradigm. This is of special relevance when taking into account the caspase-8-mediated mechanisms of apoptosis described for DKO MEFs subjected to long times glucose starvation [130].

  In deprivation models, autophagy is in charge of maintaining functional ATP levels. In this scenario, impairment of autophagy would accelerate energy depletion and promote cell death as reported previously by other authors [46]. This hypothesis is also reinforced by the morphological observation in HeLa cells, a cell line characterized by a slower kinetics of death. In HeLa cells we observed a concomitant apoptotic and necrotic morphology at long times of SM (data not shown).

- **A rapid and robust caspase-activation increase leads to necrotic cell death in autophagy-incompetent cells.** Our results shows that at long times of SM, autophagy-incompetent cells (WT MEFs cells treated with 3-MA and *Atg5* MEFs -/-) display a more rapid kinetics of caspase activation when compared to autophagy proficient cells (paper 1, Fig. 3D and 4C). We propose that such abrupt increase could promote caspase-dependent necrotic cell death in cells with impaired autophagy. Accordingly, autophagy would promote survival by avoiding the engagement of this form of cell death. A form of Caspase-dependent necrotic cell death have been for example reported in neuroblastoma cells subjected to Chelerythrine. Chelerythrine induces an early and robust caspase-activation which leads to regulated necrosis. In this model, necrotic cell death can be turned into apoptosis by modulating ROS level with thiolic antioxidants [131]. Upon SM, we observed an increase of ROS production over time, but we never explored the protective role of thiolic antioxidants.

- **Lethal gain of function of a compensatory form of autophagy.** Long-term inhibition of macroautophagy can lead to compensatory upregulation of CMA[132] or of non-canonical forms of autophagy [87, 86.] After long times of SM we report a slight decrease of p62 in autophagy incompetent cells (3-MA treated WT MEFs cells and *Atg5* $^{-/-}$ MEFs) consistent with an engagement of alternative forms of autophagy (Paper 1, 4A, 5B). Accordingly, we propose that an engaged compensatory form of autophagy would acquire a lethal gain of function and promote greater cell death than in the presence of canonical autophagy. To our knowledge though, no reports exist in this direction, while protective function of compensatory CMA has been for example documented in *Atg5* $^{-/-}$ MEFs subjected to pro-apoptotic triggers such as ROS-generating menadione and UV light [132].

2 Discussion paper 2

"2-Phenylethynesulfonamide (PES) uncovers a necrotic process regulated by oxidative stress and p53"

This paper underscores the fine interrelation of ROS and p53 in triggering regulated necrosis. Because of its high immunogenic nature, necrotic cell death is increasingly acquiring attention as a possible alternative to the traditional pro-apoptotic chemotherapeutic compounds. The biological effect of PES makes it a good candidate for therapeutical administration, since *in vitro*, PES is reported to not harm non-neoplastic cells. *In vivo* though, PES is reported to potentially affect kidney and liver. However, the possible combination with BSO (Paper 2, Fig. 4D) could markedly reduce these adverse effects. PES is reported to impair capital homeostatic mechanisms: the molecular chaperone HSP70, the lysosomal-degradative autophagy, UPS but also to block the translocation of p53 to the mitochondria. In addition to what has been discussed in the "discussion section" of each publication, we propose the following topics:

Role of autophagy in PES-induced necrosis

Cells subjected to PES display an early increment of LC3-II, a sign of lysosome blockage when accompanied with steady levels of LC3-II in the presence of bafilomycin A_1 or chloroquine (data not shown). Accordingly, ultrastructural images of PES-driven necrosis shows the abundant presence of autophagosomes containing cytoplasm and organelles. These two facts together are pointing out to a blockage of the maturation step [133]. Autophagy-incompetent cells (3-MA and Spautin-1 treated cells, Paper 2, Fig. 3A) are more sensitive to PES. In the same line, *Atg5* $^{-/-}$ MEFs are more prone to undergo PES-driven cell death (data not shown). To understand the protective role of autophagy in the cytotoxic profile of an autophagy-suppressor (PES), we propose a protective role of autophagosomes sequestering damaged cell components and thus, avoiding PES-driven necrosis. This hypothesis is consistent with the role of autophagy counteracting proteotoxicity, formation of aggregates and ROS. In addition to its role increasing the proteotoxicity, PES is a powerful inducer of ROS. In this sense, a mild increase of ROS triggers a specialized form of autophagy, termed mitophagy [134], which would sustain viability by buffering oxidative stress through the clearance of damaged mitochondria. These two cellular responses to PES would be at the origin of PES-mediated increase of autophagic markers (LC3-II).

ROS-p53 partnership in PES-driven regulated necrosis

In context-specific situations, both ROS and p53 can trigger either apoptosis or necrosis according to the magnitude of the stimulus. PES treatment induces an early ROS increase and an upregulation of p53. By using the fluorophore dichlorofluorescin diacetate (DCFDA), we observed that PES has direct oxidant properties (data not shown). The upregulation of p53 is dependent on ROS since the presence of thiolic-antioxidants (i.e. DTT) strongly diminishes p53 levels (Paper 2, Fig 7A). However, the mechanism of p53 upregulation remains a subject of debate. In this sense, we anticipate two possibilities: first, the upregulation of p53 is a consequence of ROS-dependent DNA damage and the subsequent engagement of the DNA damage response (DDR). Second, ROS-dependent upregulation of p53 is a consequence of post-translational modifications occurring via the engagement of protein kinases, such as p38α, MARK (mitogen activated protein kinase), ATM (ataxia-telangiectasia mutated protein) and ERK (extracellular signal-regulated kinases) [135].

In our experiments, we observed that the inactivation of p53 by genetic or pharmacological strategies resulted in diminished ROS, thus establishing a clear link between p53 and ROS. The predominant localization of p53 in a chromatin-enriched fraction and the upregulation of p21 are two characteristics of PES-treated cells (Paper 2, Fig. 6A and 6B). These facts point out to a transcriptional function of p53. In this sense, we propose the following possibilities to explain the transcriptional mechanisms of p53 promoting ROS:

- p53-dependent metabolic changes. Active p53 induces the transcription of "TP53-induced glycolysis and apoptosis regulator" (TIGAR) and cytochrome oxidase 2 (SCO2) [136]. TIGAR redirects glucose metabolism towards the pentose phosphate pathway (PPP) by blocking phospho-fructokinase-1 (PFK1) with a neat increase of NADPH, which in turn instigates ROS generation. The induction of SCO2 – an essential element for maintaining the function of the mitochondrial respiratory chain – stimulates the oxidative phosphorylation.
- The upregulation of p53 is reported to generate ROS through the transcription of genes such as, BAX, PUMA, NQO1 (quinone oxidoreductase, PIG3) and proline oxidase (POX, PIG6), which function as uncoupling mitochondrial proteins. In addition, p53 suppress the transcription of genes from the enzymatic antioxidant arsenal, such as manganese superoxide dismutase (MnSOD), PIG12, Aldehyde Dehydrogenase 4 (ALDh4), Glutathione peroxidase (GPX) [135].

PES-driven necrosis in the context of other p53-dependent necrotic cell death models

p53 is implicated in the necrotic cell death from two other models. WT and *Bax* $^{-/-}$, *Bak* $^{-/-}$ (DKO) MEFs subjected to H_2O_2 undergo CYPD-dependent regulated necrosis. In order to trigger necrosis, p53 needs to translocate to the mitochondria where it interacts with CYPD, regulating the opening of MPTP. Despite this model matches our observations (p53 driving necrosis), this possibility is excluded by two reasons: first, PES was initially identified as a pharmacological inhibitor of p53-translocation to mitochondrion and second, cyclosporine A (an inhibitor of CYPD) is unable to rescue WT MEFs from PES-driven necrosis (data not shown). Yet in another analogous system, MEFs DKO succumb to etoposide by undergoing a form of necrotic cell death largely dependent on p53-mediated transcription of Cathepsin Q, in cooperation with ROS-driven DNA damage [137]. HSP70, the molecular target of PES, is known to regulate the lysosomal integrity [138]. In a previous publication, the lysosomal permeabilization and release of cathepsin D were the molecular triggers of PES-driven necrotic cell death. In this setting inhibition of cathepsin D proteolytic activity by pepstatin A rescued the viability of PES-treated cultures. The inhibition of aspartyl peptidases such as cathepsin D with pepstatin A or with PepA-P (pepstatin A penetratin, a much soluble analog of pepstatin A) was unable to rescue ontogenically different cell lines from PES-driven toxicity. In addition, the lack of a consistent protection conferred by Z-FG-NHO-Bz (a general inhibitor of cysteine cathepsins such as cathepsin B, cathepsin L and cathepsin S) was suggesting that PES-driven necrosis was independent of cathepsins.

3 Discussion paper 3

"Cell death induced by 2-phenylethynesulfonamide uncovers a pro-survival function of BAX"

In this paper we uncovered a pro-survival function of BAX in the context of PES-driven necrotic cell death.

Employing isogenic knock-out MEFs, we evaluated the contribution of pro-apoptotic effector BAX and BAK in PES-driven necrosis. Surprisingly, BAX-incompetent MEFs (*Bax* $^{-/-}$ and DKO^{-}) were more prone to die when compared to the WT counterpart (Paper 3, Fig 2A). Accordingly, PES cytotoxic effect was dampened by the reintroduction of functional BAX in DKO MEFs (Paper 3, Fig. 3A). Morphologically, PES-treated WT MEFS showed the presence of images of mitochondrial fusion. As suggested by this ultrastructural characteristic, we surmised that mitochondrial dynamics modulation could be the key mechanism for BAX pro-survival function. In this sense, BAX is reported to promote fusion by its interaction with mitofusion-2 (MFN2). For this effect, BAX needs to shuttle from the cytosol to mitochondria and interact with MFN-2 [139]. This is confirmed by the loss of protective effect when a mutated form of BAX (BAXS184E, unable to translocate to the mitochondria) is employed (Paper 3, Fig. 3A). Mdivi-1 is an inhibitor of fission, which acts by targeting the GTPase activity of Drp1, thus promoting fusion. The Mdivi-1 protection in BAX-competent cells facing PES, corroborates the protective effect of fusion in PES-driven necrosis. However, this protection, though still active, is much reduced in the background of BAX-deficient cells. Thus indicating that BAX is partially mediating Mdivi-1-protective effects.

This manuscript suggests that an impairment of the pro-apoptotic protein BAX is predisposing cells to undergo necrotic cell death. We find several manuscripts that are against this idea. Some authors have recently reported a pro-necrotic function of BAX in the context of CYPD-regulated necrosis. *Bax* and *Bak*-null mouse were protected from myocardial infarction. Moreover DKO MEFs subjected to ionomycin - a Ca^{2+} ionophore described to elicit MPTP opening - were protected facing necrotic cell death. Moreover, it was demonstrated that necrotic cell death was promoted by BAX-dependent fusion [140]. The apparent contradiction with our results can be explained by the different mechanism of action involved in Ionomycin and PES-driven cell death, since PES-induce necrotic cell death doesn't seem to involve the opening of MPTP, as mentioned before.

VIII CONCLUSIONS

SM-driven cell death.

1. All the studied cell lines engage canonical autophagy, mediated by ATG5 and Beclin-1, upon starvation media (SM) treatment.
2. SM induced caspase-dependent intrinsic apoptotic cell death, since BAX and BAK proteins deficiency implied complete protection after 24h.
3. At short time of SM treatment, autophagy incompetent cells (*Atg5* $^{-/-}$ and 3MA treated cells) displayed reduced MOMP and release of cytochrome C, which is translated into inhibition of cell death.
4. At long time of SM treatment, autophagy incompetent cells showed increased ratios of cell demise.
5. Autophagy-incompetent cells were more resistant facing pro-apoptotic compounds (etoposide, staurosporine and camptothecin).

PES-induced cell death:

1. PES triggers a caspase-independent non-apoptotic cell death. Necrostatin-1 lack of protection excluded RIP1-dependent necroptosis.
2. Autophagy is protective facing PES as proven by both pharmacological and genetical autophagy ablation.
3. ROS are a crucial element in PES cell death. This is proven by the early ROS biogenesis and the protection conferred by thiolic antioxidant agents.
4. p53 contributes to PES-induced cell death in a transcriptional-dependent manner. Accordingly, p53 was predominantly in the chromatin-enriched fraction.
5. ROS and p53 are engaged in a positive feedback loop leading to the final cell death outcome.
6. PES-induced cell death is more prominent in BAX protein deficient cells, thus underscoring a putative pro-survival function of BAX.
7. PES-induced cell toxicity is dampened by induction of mitochondrial fusion, which is promoted by BAX protein.

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